REVIEW ARTICLE

Molecular Cytogenetics in Diagnostics of Malignant Diseases

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ABSTRACT

Michalová K., Zemanová Z.: Molecular Cytogenetics in Diagnostics of Malignant Diseases
Malignant cell transformation can be defined as a series of progressive genetic events which take place in one cell clone in limited number of specific genes. These genes could be oncogenes and/or tumor suppressor genes (antioncogenes, recessive oncogenes). Each change, whether or not it is associated with the initiation or progression of cancer, can be related to a chromosomal rearrangement. If the aberration is above the limit of the light microscope sensitivity, it should be detected by classical cytogenetic techniques. Therefore it was hypothesized that the molecular characteristics of chromosomal rearrangements will lead to identification of genes with a pivotal role in cancerogenesis. And indeed, genes important for the origin of tumours were ascertained in recurrent chromosomal breakpoints. Until now more than 1 800 breakpoints have been identified. Oncocytogenetics has developed at a remarkable pace since the introduction of molecular methods with higher sensitivity (100 kb). We present a short review of molecular cytogenetic methods with a survey of specific recurrent rearrangements; translocations and deletions of chromosomes in several malignancies and their prognostic value is given.
Key words: chromosomal rearrangements, malignant tumours, leukemia, molecular cytogenetics. *Mi.*

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A malignant cell is different from a normal one due to constant and uncoordinated cell division caused by loss of the signals that control this function. A malignant cell transformation can be defined as a series of progressive genetic events which take place in one cell clone in limited number of specific genes. These genes could be oncogenes and/or tumour suppressor genes (antioncogenes, recessive oncogenes). Each change, whether or not it is associated with initiation or progression of cancer, can be related to a chromosomal rearrangement. If the aberration is above the limit of the light microscope sensitivity, it should be detected by classical cytogenetic techniques. Therefore it was hypothesized that the molecular characteristics of chromosomal rearrangements will lead to the identification of genes with pivotal role in cancerogenesis. In fact, during the last decade a lot of genes which play an important role in a development of malignant tumours have been localized by cytogenetic methods. It was found that they are mainly cell oncogenes (protooncogenes) which do not cause cell malignant transformation in normal circumstances. In the healthy cell oncogenes code proteins, regulating a normal proliferation of the cell, and only their mutation or atypical activation leads to loss of control and change of a normal cell into a malignant one (1).

ONCOCYTOGENETICS

Tumour cytogenetics deals with the study of acquired chromosomal changes in benign and malignant tumours. Although until 1970 only classic, homogenous staining of chromosomes was applied, numeric aberrations or large structural rearrangements of chromosomes had been described in tumours before 1972. The most important finding for oncocytogenetics was identification of the Philadelphia chromosome (Ph chromosome) by Nowell and Hungerford (1962) in patients with chronic myeloid leukemia (CML); as a stable marker, and its discovery stimulated a large cytogenetic research of malignant cells. Incidentally, finding of Ph chromosome also supported the hypothesis that each variant of tumour would have own typical marker chromosome (2). Unfortunately, this hypothesis was not confirmed; on the contrary, the Ph chromosome remained for over 15 years the only specific aberration found in tumour cells. Rowley (1973) ascertained by G - banding technique that the Ph chromosome originated by reciprocal translocation between chromosomes 9 and 22 (3) described later on according to ISCN as t(9;22)(q34;q11). A further specific cytogenetic aberrations were detected by banding of chromosomes, and oncocytogenetics became an important laboratory discipline specially when diagnosis of specific types of leukemias, lymphomas and solid tumours is established (1). Because of easier sampling and processing of bone marrow cells and/or peripheral blood for cytogenetic analyses, hematooncological diseases were more often examined in the past and now represent more than two thirds of cases quoted in catalog of chromosomal aberrations in malignant cells which is managed by Mitelman (http://cgap.nci.nih.gov/Chromosomes/Mitelman/recurrent/aberrations).

Since 1970 with introduction of banding techniques more than 1 800 of chromosomal breakpoints have been described in tumours. These refer to aberrations which are recurrent and non-random. We can suppose that some of them are involved in a

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Tab. 1. Selected specific rearrangements of chromosomes and their prognostic value

Chromosomal change	genes	Clinic characteristic	prognosis
t(9;22)(q34;q11)	BCR/ABL	CML, ALL	in ALL poor
t(8;21)(q22;q22)	AML1/ETO	AML M2	good
t(15;17)(q22;q12)	PML/RARA	AML M3	good
inv(16)(p13q22)	CBFB/MYH11	AML M4	good
t(8;16)(p11;p13)	MOZ/CBP	AML M5	
t(1;7)(q10;p10)	?	MDS	
del(5)(q31)	?	MDS	good
+8	?	AML M2, M4, M5, MDS	inter-mediate
t(6;9)(p23;q34)	DEK/CAN	MDS, AML M2, M4	poor
t/del(11)(q23)	MLL	AML, MDS, ALL	poor
t/del(12)(p12)	TEL	AML, ALL, MDS	poor
del(20)(q12)	?	PV, AML	
t(1;19)(q23;p13)	PBX1;E2A	ALL L1	poor
t(4;11)(q21;q23)	AF4;MLL	ALL L1, L2	poor
t(12;21)(p12;q22)	TEL/AML1	B-ALL	good
del(6)(q)	?	ALL L1,L2, CLL	
t(8;14)(q24;q11)	MYC;TCRA/TCRD	B- or T-ALL	inter-mediate
t(14;18)(q32;q21)	IGH/BCL	ALL L2, L3, B-CLL	
t(12;16)(q13;p11)	FUS/CHOP	liposarcoma	
t(X;18)(p11;q11	SYT/SSX	Synovial sarcoma	
t(2;13)(q35;q14)	PAX3/FKHR	rhabdomyosarcoma (alveolar)	poor
t(12;15))p12;q25)	ETV6/NTRK3	Fibrosarcoma	
inv(10)(q11q21	RET/PTC	papilar carcinoma of thyroid	
t(X;1)(p11;q21)	PRCC/TFE3	Carcinoma of kidneys	

Tab. 2. Selected commercially available DNA probes for diagnostics of malignant tumours

Type of tumour	Type of probe	Localization
Breast cancer	Locus specific	17q11.2-q12
	Her-2 Neu	
Carcinomas	Locus specific c-myc	8q24.12-q24.13
Prostate cancer	Locus specific androgen receptor gene	Xq12
Bladder cancer	multicoloured kit centromeric	Cen 3, 7, 9, 17, 9p21
	3, 7, 9, 17, locus specific 9	
chronic myeloid leukemia	locus specific BCR/ABL	9q34 and 22q11
acute myeloid leukemia M2	locus specific AMLl1/ETO	8q22 and 22q22
acute lymphocytic leukemia	locus specific TEL/AML1	12p12 and 21q12
myelodysplastic syndrome	locus specific probe for deletion 5q	5q31
acute leukemia	locus specific two colour	11q23
	break apart probe MLL gene	
myelodysplastic syndrome and leukemia	centromeric probe chromosome 7 and/or 8	cen 7 and cen 8
chronic lymphocytic leukemia	Panel of probes	11q22.3, 17p13, 13q14, 13q34, cen12
multiple myelom, lymphomas	locus specific, two colour break apart IGHV	14q32 part

process of malignant growth initiation and the others in the progression of the disease. Each year the number of new chromosomal changes of malignant tumours studied by molecular methods is described. As already mentioned above there exist free databases dealing with chromosomal aberrations of malignant cells as for example Mitelman's catalog of chromosomal aberrations, or Encyclopedia of genetics and cytogenetics in oncology and hematology (http://www.infobiogen.fr/services/chromcancer) as a free database on the internet which offers cytogenetic and clinical findings in malignant diseases and which is used by geneticists and physicians interested in oncology. In Mitelman's catalog about 50 000 chromosomal findings in malignant cells was assembled from scientific papers published until the end of 2005, for example.

The most specific aberrations and their predictive value in the

different types and subtypes of leukemias and lymphomas are shown in Tables 1 and 2 of this article.

CONVENTIONAL AND MOLECULAR CYTOGENETICS

The rapid development of human cytogenetics was promoted by introduction of banding techniques of chromosomes, by the new cultivation systems and growth medias for tissue culture and also by synchronization of the cell divisions of malignant cells cultivated *in vitro*. The quality of chromosomal preparations has improved, sensitivity of methods has increased and international nomenclature ISCN started to be used (4). Other previously unidentified rearrangements, as are translocations of small parts of chromosomes, inversions, duplications, deletions and insertions, have been detected, and some of them turned out to be a specific marker of a specific tumour's variant. Most of them were detected by methods of molecular DNA analyses only.

Fluorescence in situ hybridization (FISH)

Methods of hybridization *in situ* are based on the ability of the DNA single-chain to link with complementary parts of the target DNA, which is fixed on a microscopic slide (5). In fact, non-radioactively labelled DNA probes are used, and localization of probe is performed by indirect or direct imunofluorescence in a fluorescence microscope. Size of the DNA fragment explored by molecular methods is approximately 200-300 kb. The method of FISH is successfully and frequently used in clinical cytogenetics and oncocytogenetics when surveying cell karyotype in metaphase or interphase. By FISH method it is possible:

- to determine numeric and exactly identify structural hereditary or acquired chromosomal aberrations and marker chromosomes;
- to map genes and the DNA sequences, observe their deletion or amplification;
- to locate genes on chromosomes;
- to map and localize genes in comparative studies of DNA in different species of plants, animals and human.

Very special and advanced is technique called interphase FISH – I-FISH, because it makes possible to observe numerical and structural aberrations of chromosomes in interphasic, non-dividing nuclei of malignant cells. Using this method it is possible to determine:

- quantitative representation of pathological clone in sample of a malignant tissue;
- amplification, translocation or deletion of single genes;
- detection of a minimal residual disease or early relapses in leukemias and malignant solid tumours;
- monitoring of result of chemotherapy and/or bone marrow transplantation in patients with leukemias;
- detection of cells which are included in a neoplastic process.

Identification of fluorescence signal is possible directly from the fluorescence microscope. Cytogenetic analysis could be computerized, using microscope with high performance CCD (charge coupled device) camera and linked to a computer with special software for FISH. The sensitivity of the method increases by one order of magnitude at least and at the same time more fluorochromes can be used for simultaneous evaluation. A computer analysis of an image enables the quantitative processing of acquired data, measurement of distances between single signals and the creation of real pictures from more records. Automatic screening of microscopic slides with special software for different FISH techniques and conventionally stained microscopic slides is possible as well.

DNA probes

The probes used for FISH are of different types, and they can be easily combined. There are probes which hybridize with *specific chromosomal structures*. These are usually centromeric parts of alpha satellite sequences of the DNA. The satellite DNA is chromosomes specific, highly repetitive and tandem arranged, size reaches up to 4 000 kb. Similarly, as for centromeric parts, there are DNA probes for subtelomeric and telomeric regions of human chromosomes and all these probes can be used for examination of interphase cells (I-FISH).

In addition, there are locus specific probes (LSI) which hybridize with the single sequences of DNA. They are usually genomic clones which differ in size depending on the vector. The clone vector can be plasmid (500 bp-5 kb), cosmid (20-50 kb), bacteriophage lambda (8-15 kb) or artificial yeast chromosomes (YAC clones 50-1 000 kb), artificial bacterial clones (BAC), P1 clones and other vectors. LSI probes can be used for I-FISH technique as well.

To prove translocations and insertions we use special probes which hybridize with *multiple chromosomal sequences*. These probes are usually made from specific chromosomal libraries of the DNA and whole chromosomes are labelled (chromosome painting). Painting probes can be also acquired by micro dissection of a specific part of chromosome from fixed preparations; then the acquired DNA is amplified by DOP-PCR (polymerase chain reaction with degenerate oligonucleotides). Painting probes cannot be used for screening of interphase nuclei because the signal of whole single chromosome is in interphase dispersed as chromosomes are despiralized.

Comparative genomic hybridization (CGH) and matrix CGH

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique which enables the detection and mapping of relative numbers of copies of single sequences between different genomes (6). The normal and examined DNA are differently labelled by fluorochromes and they are simultaneously co-hybridized on microscopic slide with normal metaphase chromosomes. Chromosomes with amplification or loss of the DNA sequences such are highlighted by a different color. This is caused by change of a rate of a signal intensity of both fluorochromes which are measured along the normal chromosomes. Using special software the signal intensity is measured in at least 10 mitoses and regions with genome rearrangements as deletion or amplification are easily identified. CGH cannot be used to assess reciprocal translocations, inversions and insertions in which the rate of the DNA sequence copies has not changed M*atrix CGH* is working on the same principle as CGH except that examined labelled DNA is hybridized on microchips instead on microscopic slides with mitotic chromosomes.

Multicolour karyotypes of human chromosomes (mFISH and mBAND)

Since 1996 it is possible to distinguish each single pair of autosomes and sex chromosomes by different colour. The technique is called multicolour FISH (mFISH) and it is another modification of basic FISH method in which each single pair of autosomes and sex chromosomes are combinatorially labelled by combination of five fluorochromes and then with the help of CCD camera, computer and special software digitalized image is analyzed. The mFISH method is very appropriate for determination of origin of marker chromosomes, identification of cryptic chromosomal rearrangements as insertions, balanced and unbalanced translocations. On the similar principle works a method of multicolour banding with a high resolution (mBAND) which can be used for study of complex rearrangements of karyotype and determination of origin of marker chromosomes as well. The same

fluorochromes as for mFISH are used to label microdissected parts of one chromosome. With mBAND technique we can exactly locate breakpoints on chromosomes and detect extent of translocations, deletions or any structural rearrangement. Probes for mFISH and mBAND (for every autosome and X and Y chromosomes) are commercially available and the techniques are used for routine examinations and research as well.

INDICATIONS FOR MOLECULAR CYTOGENETIC EXAMINATION

It is recommended to use FISH methods strictly on the basis of results of classic cytogenetic examinations and according to the diagnosis of the patient. I-FISH method as a screening technique is applied in those patients where failure of cultivation of the cells for the cytogenetic examination appeared or in a case of monitoring of cytostatic treatment in oncohematologic patients, including those with bone marrow transplantation. In addition, I-FISH is used for examination of bone marrow cells for amplification of some genes, as an example can be quoted amplification of N-myc oncogene in work-up diagnosis of neuroblastoma, amplification of c-myc in solid tumours, amplification of Her-2neu in breast cancer, amplifications of genes and chromosomes in diagnostics of bladder tumours, brain tumours and many other cases. As it was already said CGH can be used in monitoring of amplifications and deletions of genomes of malignant cells and this approach brought recognizable progress into cytogenetics of solid tumours. We suppose that chip technologies including matrix CGH will be used more and more goal-directed in the near future.

Deletion of chromosomes

Many deletions of a different extent were detected by classic banding techniques in chromosomes of malignant cells. Molecular cytogenetic methods helped in identification of cryptic changes and breakpoints. As an example we can mention brain tumours which were studied intensively during the last few years and we learned about the role of new diagnostic and prognostic indicators, some of them are mentioned below.

In diffuse gliomas deletion of gene p53, which is normally located on short arms of chromosome 17 in region 17p13.1, is often present. Gene p53 belongs to a group of tumour-suppressor genes and is the most frequently mutated in all different types of malignant diseases. It codes information for protein of molecular weight 53 kDa which plays an important part in the regulation of the cell cycle, cell response to DNA damage and regulation of apoptosis. The loss of genomic information (deletion) or mutation of gene p53 were detected in 30-40 % astrocytomas of all three degrees and it shows that elimination of function of this gene is often a critical event in the origin of astrocytomas (8, 9).

Change from the low-grade astrocytoma to anaplastic is often combined with deletion of short arms of chromosome 9 in the region 9p21. where is located other tumor suppressor gene p16 (CDKN) , which has an important role in regulation of cell cycle (9). Protein p16 is blocking activity of cyklin-dependent kinases (Cdk4/6) as well and prevents their binding on cyklin D. Deletion of p16 was found in approximately two thirds of all gliomas of higher degrees (anaplastic astrocytoma, primary or secondary glioblastoma).

In approximately one third of patients with high-grade astrocytoma deletion of RB1 gene was found. This tumour

Tab. 3. Specific chromosomal aberrations in different subtypes of diffuse gliomas

	Type of diffusional glioma	WHO grade	Chromosomal aberrations
astrocytomas	Low grade astrocytoma	II	trisomy 7, aneuploidy
	anaplastic astrocytoma	III	Deletion of gene *p53*, deletion of gene *p16*, deletion of gene *RB1*, aneuploidy
	glioblastoma multiforme - primary	IV	Amplification of gene *EGFR*, deletion of gene *p16*, deletion of gene *RB1*, deletion of gene *p53* (rarely), monosomy 10, aneuploidy
	glioblastoma multiforme - secondary	IV	Deletion of gene *p53*, deletion of gene *p16*, deletion of gene *RB1*, monosomy 10, aneuploidy
oligodendrogliomas	Low grade oligodendroglioma	II	Deletion of region 19q13.3, aneuploidy
	anaplastic oligodendroglioma	III	deletion 19q13.3 / deletion 1p36, aneuploidy (finding of combined deletion is a result of a good response to chemotherapy)

Tab. 4. Summary of chromosomal changes detected in bladder cancer

Saran et al., 1996	deletion 3p and 17p
Al-Sukhum et al., 2003 (12)	deletion 4p, 17p and isochromosome i(5)(p)
Khaled et al., 2004 (13)	loss of chromosome Y
Kim et al., 2005 (14)	deletion 11p, 8p, polysomy 8q and 1q
Okamura et al., 2004 (15)	aneuploidy 9 a 17
Panani et al., 2004 (16, 17)	monosomy 9, polysomy 11, structural aberrations 3p12, 6q23, 11p15, 14q32 and 19q13, i(8)(q), i(17)(q) and i(6)(p)
Steidl et al., 2002 (11)	aneuploidy 1, 8, 9, 11, 17, 1p36, 8p23, 9p21, 11q13, 17p13

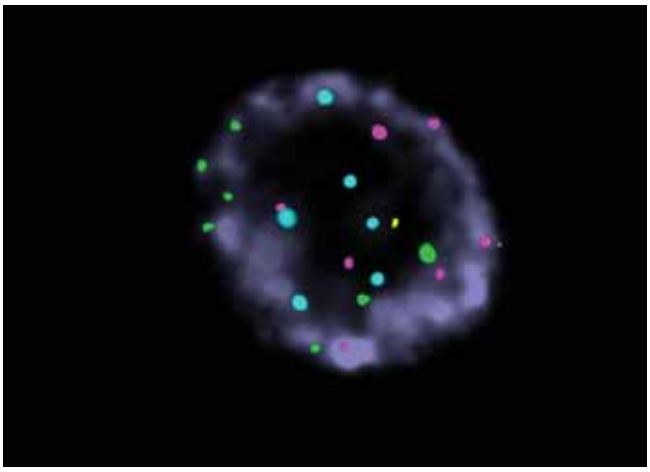

Fig. 1. Interphase cell of bladder cancer with polysomy of chromosomes 3 (pink signal), 7 (blue signal) and 17 (green signal)
Commercially available kit of the DNA probes from Abbott-Vysis (*UroVysion Bladder Cancer Recurrence Kit*) was used to fluorescence in situ hybridization.

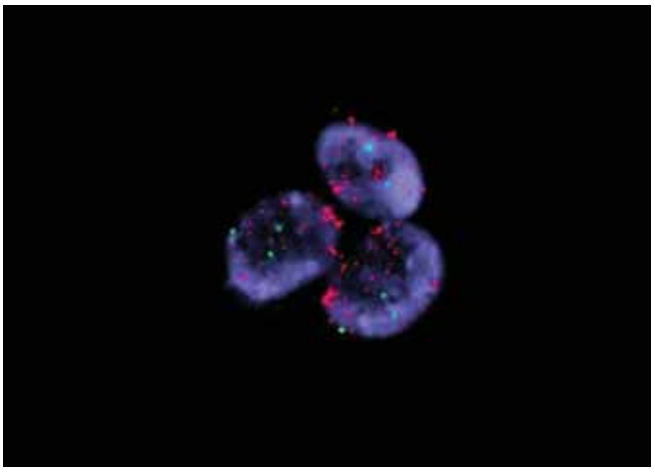

Fig. 2. Amplification of gene EGRF (red signal) and trisomy of chromosome 7 (green signal) in interphase cells of a patient with the primary glioblastoma
The probe *LSI EGFR (labeled Spectrum Orange) and trisomy 7(labeled Spectrum Green)* from Abbott-Vysis was used for I-FISH

suppressor gene is located in the region13q14 and is coding protein of molecular weight 107 kDa. During phosphorylation of RB protein it comes on release of the transcriptional factor E2F with which RB protein forms a complex and activates genes which are necessary for cell proliferation. Deletion of RB1 was found at first in somatic cells of patients with familial or sporadic retinoblastoma. However, later on it was ascertained in other types of solid tumours (for example osteosarcoma) and hematological malignant diseases, for example chronic lymphocytic leukemia (CLL) and multiple myeloma (MM). In both diseases presence of Rb1 gene can be examined by I-FISH and the presence or absence of this oncogene is at present strong prognostic factor.

In diffuse gliomas deletions of short arms 1p and long arms 19q are quite common. The most frequent allelic losses are in the region 1p36 and non-random losses of genetic materials from chromosome 1 (esspecially telomeric part 1p) were detected in wide range of malignant diseases of different types of solid tumours, leukemias and myeloproliferative diseases. This means that in this region of chromosome 1 are located genes which are important at the origin of malignant tumour. Therefore, the existence of one or more candidate tumour-suppressor genes is expected. In Table 3 are presented the most frequent deletions found in individual types of brain tumours.

Bladder cancer is another tumour which is frequently studied by molecular genetic methods. In this tumour deletions of short or long arms of many different chromosomes were detected (Table 4). Loss 9q was ascertained in a few differentiated and in highly differentiated tumours as well. This shows that this change could be by primary symptom of genesis of the bladder cancer. The loss of heterozygosity – meaning the loss of one allele (LOH) of 17p was detected in 60 % of invasive tumours but not in superficial tumours. This indicates important role of this aberration in a progress of disease. Steidl et al. (11) concentrated on detection of chromosomal aberrations by method of I-FISH. with the use of centromeric and locus-specific probes and proved that at least in one chromosome numerical changes could be detected in 100 % of bladder cancer samples analysed.

Amplification of chromosomes

Amplification of single genes or whole chromosomes and/or their parts is a very frequent finding in malignant cells and can be one of the mechanisms leading to progress of the tumour because of higher genetic dosage (dosage effect). In the malignant disease a finding of an abundant chromosome or its part is usually indication of the progress of a malignant process. As an example can be mention clonal development in patients with chronic myeloid leukemia (CML) when, during the transition from chronic to blastic phase, relatively uniform clonal development occurs. It can appear as trisomy 8, isochromosome i((17)(q) or additional Ph chromosome which does not develop by new translocation, but by mitotic nondisjuction. In addition, in myelodysplastic syndrome (MDS) and acute leukemias new numerical or structural changes originate (amplification of chromosomes) during the progression of the disease and they are connected with a bad prognosis for the patient. In different types of solid tumours polyploidy is often described. In the case of bladder cancer it occurs polysomy of chromosomes 3, 7 and 17 (Fig. 1). In different types of leukemia and solid malignant tumours the amplification of single genes was ascertained. For example amplification of gene MML (mixed lineage gen) can be found in acute leukemias, n-myc oncogene is diagnostic indicator for neuroblastoma or amplification of gene *EGFR* in brain gliomas of higher degrees (Fig. 2).

Chromosomal translocation

Chromosomal translocations in malignant cells cause activation of existing genes or create new fusion proteins. There is usually intercellular expression: either hybrid molecules with new characters or unappropriate expressed normal proteins. At the moment more than 200 different fusion genes were identified. Some of them are very promiscuous and recombine with many different partners – for example MLL gene in acute leukemias –more than 40 partner chromosomes were identified already, some breakpoints were involved more frequently. In Table 1. are presented some of the chromosomal translocations and the fusion genes described in malignant tumors and also those translocations which involve T-cell receptors and IgH chains.

The new technologies which are recently developed and promise very specific and effective treatment of the diseases concentrating on intracellular aims, can be influenced by products of these translocations i.e. by the new fusion genes or enhanced oncogenes. It is not clear whether by translocations altered expression of oncogenes is essential for late phase of cancers, their viability and growth. Chromosomal instability of malignant cells cumulation of mutations can cause that original defect of

oncogene will harm and disturb the therapeutic effect. Although present data show that chromosomal translocations are important for pathogenesis of malignant tumours, it does not have to be in all cases.

CONCLUSION

Substantial variability of deletion and translocation breakpoints brings a lot of problems, with diversion of chromosomal rearrangements in the same group of diseases and with the exact localization of breakpoints on given chromosome. We try to find a quick and reliable method for the identification of corresponding translocations and/or deletions specific for each cancer. In addition, we try to find a cause of specific treatment to give the correct and precise therapeutic agens to each patient. Application of new sophisticated technologies for the identification of specific lesions and insurance of a products specific for given type of cancer, could move us to a period of individual, molecularly targeted therapy against malignant molecules of each single patient.

Abbreviations

ALL	– acute lymphocytic leukemia
AML	– acute myeloid leukemia
BAC	– bacterial artificial clones
CCD	– charged couple device camera
Cdk4/6	– cyklin-dependent kinase
CDKN	– cyklin dependent kinase
CGH	– comparative genomic hybridization
CLL	– chronic lymphocytic leukemia
CML	– chronic myeloid leukemia
DAPI-4,6	– diamidino 2-phenylindol
DOP-PCR	– polymerase chain reaction with degenerate oligonucleotides
FISH	– fluorescence *in situ* hybridization
FITC	– fluorescein isothiocyanate
I-FISH	– interphasic FISH
IgH	– heavy immunoglobulin chain
ISCN	– An International System for Human Cytogenetic Nomenclature
Kb	– kilobase DNA
kD	– kilodaltons
LOH	– loss of heterozygosity
Mb	– megabase DNA
MLL	– mixed leukemia lineage gene
mBAND	– multicoloured banding with high sensitivity
MDS	– myelodysplastic syndrome
mFISH	– multicoloured fluorescence *in situ* hybridization
MM	– multiple myeloma
YAC	– yeast artificial clone
PCR	– polymerase chain reaction
Ph	– Philadelphic chromosome t(9;22)(q34;q11)
PRINS	– PCR *in situ* hybridization

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