Diagnostics of Molecular Markers in Childhood Acute Leukaemia Using Biochips

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1. Introduction

Acute leukemia is a very heterogeneous disease that can be divided in two major groups according to lymphoblastic or myeloblastic origin of leukemic blast cells: acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML). ALL and AML, in their turn, are both subdivided into many subgroups with different clinical features.

ALL is more frequent in children and represents about 80% of all pediatric leukemia cases. Prognosis of newly diagnosed children with ALL has improved significantly mainly due to treatment with high-doses of chemotherapeutic drugs, but also a risk-stratification strategy and optimization of therapy. 5-year event-free survival (EFS) rates in different clinics range between 76% and 86%. Overall remission rates usually are 98% or higher (Pui et al., 2011).

Age and white cell count at diagnosis have been used to predict a prognosis in ALL for many years, having been identified in early epidemiologic studies as predictors of an outcome (Smith et al., 1996). Children aged from 1 to 9 years have the best outcomes; children and adolescents aged from 10 to 20 years have slightly worse outcomes, which is associated in part with higher incidence of T-cell leukemia and lower incidence of favorable genetic abnormalities such as TEL/AML1 and hyperdiploidy. Also, ALL blasts from older patients become more resistant to multiple antileukemic drugs than the blasts from younger children in the first decade of life (Pieters et al., 1998; Nachman et al., 2009).

Infants diagnosed at age of less than 1 year have relatively poor outcomes, which is associated with high incidence of immature pro-B-ALL phenotype and presence of MLL gene rearrangements (Hilden et al., 2006). Another biologic factor of prognostic value besides immunophenotype is a rapidity of response to the induction therapy with glucocorticoids, for instance decrease in peripheral blood blast count after a week of treatment. It has been shown by flow cytometry and molecular techniques, that a level of minimal residual disease (MRD) in bone marrow during first months of therapy may have high prognostic value and is used for stratification in many protocols (Szczepanski et al., 2001). Further risk stratification has been achieved using cytogenetic and molecular genetic characteristics of leukemia. The TEL-AML1 translocation and hyperdiploidy were found to predict a good prognosis; therefore they allow relative therapy reduction in carriers (Pui et al., 2000). Philadelphia-chromosome-positive ALL (Ph+−ALL) is associated with a poorer prognosis...
and application of novel therapies may significantly improve clinical outcome (Arico et al., 2000). Introduction of tyrosine kinase inhibitors has completely changed therapy strategy for chronic myeloid leukemia (CML), potentially it may be a treatment of choice for Philadelphia positive ALL. Historically, the children with Ph+- ALL have been transplanted in their first complete remission (Davies & Mehta, 2010).

Acute myeloid leukemia (AML) is defined as a hematologic malignancy in which more than 20% of nucleated cells represent myeloid blasts by morphology and immunophenotype. Despite apparent phenotypic uniformity, it has become increasingly clear that AML is a heterogeneous group of neoplastic diseases (Watt & Bagg, 2010). To great extent, the heterogeneity is based upon its genetic complexity. The traditional parameters such as clinical features, blood counts, morphology, cytochemistry, immunophenotype are keeping their position in clinical evaluation, but genetic approaches are now firmly established as the central component in diagnostics and classification of AML. There are different, but recurrent, structural and numeric cytogenetic abnormalities, translocations, inversions and derivative chromosomes (Mitelman et al., 2011). Three broad prognostic groups in AML with either favorable, intermediate, or adverse prognosis are identified by specific cytogenetic abnormalities (Grimwade & Hills, 2009). Also there is a growing number of acquired gene mutations which are essential to pathogenesis of AML (Gaidzik & Dohner, 2008). In addition, aberrant gene expression and copy number variations have recently been recognized as a common phenomena that underlie malignant transformation (Eklund, 2010). The average EFS rate in AML is significantly lower comparing with ALL and usually ranges from 40% to 63% in most successful clinical trials, thus stimulating the search and implementation of new approaches to the treatment based on molecular genetic markers. Molecular technologies continue to evolve and provide more profound comprehension of leukemia pathology. Many of them have rapidly moved into clinical laboratories, while others remain as important discovery tools. Eventually, molecular genetic approaches will play the leading role in future leukemia practice.

1.1 Clinically relevant genetic lesions in ALL
The World Health Organization (WHO) provides current diagnostic criteria for ALL as a precursor B-cell acute lymphoblastic leukemia (B-ALL) or as a precursor T-cell acute lymphoblastic leukemia (T-ALL) (Brunning et al., 2001). Of the annually diagnosed ALL cases in different countries, approximately 80–85% have the B-ALL phenotype, and remainder displays the T-ALL phenotype. This classification scheme does not subdivide ALL into molecular subtypes, but most often, ALL is subtyped and studied on the basis of particular underlying genetic abnormality. The genetic defects in ALL include chromosomal translocations that deregulate gene expression or create novel fusion genes, numerical chromosome copy number aberrations (especially hyperdiploidy), and gene-specific mutations (Teitell & Pandolfi, 2009).

Hyperdiploidy (more than 50 chromosomes per leukemia cell) is found in approximately 25% of children who have B-lineage ALL. This genome abnormality is associated with a favorable outcome, especially when extra copies of chromosome 4, 10 or 17 are presented (Heerema et al., 2000). The hyperdiploid ALL cells are highly sensitive to cytostatics and L-asparaginase, and accumulate high amounts of methotrexate derivates, so they are easily subjected to apoptosis (Kaspers et al., 1995).

The TEL/AML1 (ETV6/RUNX1) fusion accounts for approximately 20-25% of cases and also is associated with a favorable outcome. It is formed by fusion of TEL gene on the
chromosome 12 encoding a member of the ETS family of transcription factors and \textit{AML1} gene on the chromosome 21, a transcription factor gene encoding a part of core-binding factor (CBF). The \textit{TEL/AML1} fusion probably inhibits transcription activity of normal \textit{AML1} gene involved in proliferation and differentiation of hematopoietic cells. The t(12;21) is cryptic and cannot be identified by conventional cytogenetics. It is associated with high sensitivity to chemotherapeutic drugs, especially to L-asparaginase, though the mechanism of the sensitivity is unclear (Ramakers-van Woerden et al., 2000). Also the sensitivity to other drugs especially anthracyclines and etoposide has been reported (Frost et al., 2004). Both hyperdiploidy and \textit{TEL/AML1} translocation occur mainly in children younger than 10 years with common or pre-B-ALL.

Abnormalities of a mixed lineage leukemia (\textit{MLL}) gene on the chromosome 11q23 occur in approximately 80% of infants with ALL, but only in 2% of children above age of 1 year. All types of \textit{MLL} gene rearrangements, such as \textit{MLL/AF4} derived from t(4;11), \textit{MLL/MLLT1(ENL)} derived from t(11;19), and \textit{MLL/MLLT3} derived from t(9;11), are associated with a poor outcome in infants (Pieters et al., 2007); but in older children the poor outcome likely remains only for \textit{MLL/AF4} fusion gene (Pui et al., 2002). The fusion products involving \textit{MLL} are associated with an aberrant expression of \textit{HOX} genes, which are involved in early embryogenesis and may influence normal development of hematopoietic stem cells (Armsrong et al., 2002). The blast cells with \textit{MLL} rearrangements are highly resistant to glucocorticoids and L-asparaginase (Dordelmann et al., 1999; Ramakers-van Woerden et al., 2004). These cells however, show a noticeable sensitivity to nucleoside analogue cytarabine, that is probably related to high expression of a membrane nucleoside transporter \textit{ENT1} (Stam et al., 2003).

The translocation t(9;22) results in a fusion between \textit{BCR} gene on the chromosome 22 and \textit{ABL} gene on the chromosome 9 leading to over expression of the abnormal ABL tyrosine kinase, thus increasing proliferation and decreasing apoptosis (Kharas & Fruman, 2005). The \textit{BCR/ABL} fusion is found mainly in common and pre-B ALL. Incidence of the \textit{BCR/ABL} increases with age from approximately 3% of children with ALL up to approximately 25% in adult ALL cases. Presence of \textit{BCR/ABL} predicts a poor outcome; more often it is associated with a poor response to prednisone and high levels of residual blasts carrying the \textit{BCR/ABL} fusion after induction therapy (Schrappe et al., 1998).

The prognostic value of genetic abnormalities in T-ALL is not so evident. The translocation t(1;14) resulted in \textit{SIL/TAL1} fusion gene is found in approximately 25% of all T-ALL cases. The translocations t(10;14) and t(7;10) occur in approximately 10% of T-ALL cases leading to activation of \textit{HOX11} gene (Graux et al., 2006). Two recently described abnormalities occur exclusively in T-ALL: an abnormal expression of HOX11L2 caused mainly by translocation t(5;14) in approximately 25% of T-ALL cases, and activating mutations in \textit{NOTCH1} gene in 50% of T-ALL cases. The mutations in \textit{NOTCH1} gene may be associated with a favorable outcome (Breit et al., 2006). Other recurrent genetic lesions occur in small subgroups of childhood ALL. The translocation t(1;19) leading to \textit{E2A-PBX1} fusion occurs in less than 5% of precursor B-ALL, mainly pre-B-ALL cases and is associated with a more aggressive clinical course (Aspland et al., 2001). Hypodiploidy (<45 chromosomes) is detected in only 1% of children who have ALL and is associated with a poor outcome (Nachman, et al., 2007).

### 1.2 Clinically relevant genetic lesions an AML

The WHO classification of AML is based on genetically defined entities of this complex disease (Swerdlow, 2008). In 2008 seven groups based on recurrent translocations (AML
with t(8;21) (q22;q22) RUNX1/RUNX1T1; AML with inv(16) (p13q22) or t(16;16) (p13;q22) CBFB/MYH11; AML with t(15;17) (q22;q12) PML/RARA; AML with t(9;11) (p22;q23) MLLT3/MLL; AML with t(6;9) (p23q34) DEK/NUP214; AML with inv(3) (q21q26.2) or t(3;3) (q21q26.2) RPN1/EVI1 and AML with t(1;22) (p13;q13) RBM15/MKL1 and two provisional groups characterized by gene mutations (AML with mutated NPM1 and AML with mutated CEBPA) were included into the classification system. These nine diagnostic entities cover about two thirds of all AML cases, while additional subtypes of AML, in which genetic factors play the central role, include myelodysplastic syndrome and therapy-related myeloid neoplasms. The latter two subtypes typically represent cases with complex karyotype, which is defined as the coexistence of three and more clonal cytogenetic aberrations (Watt & Bagg, 2010).

1.2.1 Recurrent chromosomal aberrations

The t(8;21)(q22;q22) RUNX1/RUNX1T1 is a specific genetic lesion. It occurs in approximately 8-10% of all cases of AML and usually is associated with the previously FAB-designated subtype AML-M2 (Peterson & Zhang, 2004; Swerdlow, 2008). RUNX1 and CBFB encode two components of a heterodimeric transcription complex known as core binding factor (CBF). The CBF plays an important role in regulation of normal hematopoiesis, thus the disruption of these two genes becomes obviously pathogenic. One of two major recurrent translocations involving CBF is the t(8;21), another is the inv(16). A part of RUNX1(AML1) gene on 21q22 is fused with a part of the RUNX1T1(ETO) gene on 8q22. The subsequently derived fusion protein, RUNX1–RUNX1T1 (AML1/ETO), represents a protein with inhibitory function which represses transcription of a number of important hematopoietic genes. Clinically, the t(8;21) translocation is associated with a favorable prognosis (Heerema-McKenney & Arber, 2009).

The inv(16)(p13q22) or t(16;16)(p13;q22) CBFB/MYH11 is a pericentric inversion or molecularly identical t(16;16) translocation. It represents approximately 5–10% of all cases of AML and is associated with the FAB entity of acute myelomonoblastic leukemia with eosinophilia (AML-M4Eo) (Heerema-McKenney & Arber, 2009). A part of CBFB gene is fused with a part of one of myosin heavy chain genes, MYH11 and the fusion prevents the formation of a functional CBF transcription factor. The CBFB–MYH11 can act also as a transcriptional repressor in the nucleus (Shigesada et al., 2004). Although this genetic fusion is most often seen in M4Eo, it may also be found in other subtypes of AML, including those designated by FAB as M2 and M5. The inv(16) can sometimes be missed by cytogenetics as cryptic, so molecular methods have a particularly important role in the detection of this aberration. The breakpoints in CBFB gene occur in intron 5, while in MYH11 gene breakpoints are involved seven different exons (7 through 13), yielding at least ten different fusion transcripts. The most common form, type A, accounts for approximately 90% of CBFB/MYH11 cases, while two other transcripts (types D and E) account for an additional 5%. The aberration is associated with a favorable outcome.

The t(15;17)(q22;q12) PML/RARA is observed in approximately 5–8% of all cases of AML defining an unique entity acute promyelocytic leukemia (APL) (Swerdlow, 2008). Among all acute leukemias, APL represents one with the most stable genotype–phenotype correlation, in that the presence of specific translocation can frequently be expected based upon the characteristic morphology: the classic hypergranular form (FAB AML-M3) or the microgranular variant (FAB AML-M3v). The part of PML gene on 15q22 is fused with RARA gene on 17q12 resulting in a PML/RARA gene. In the absence of retinoic acid the wild-type
RARA protein is a component of a heterodimeric transcriptional repressor complex, while binding with retinoic acid converts the RARA complex into a transcriptional activator. The chimeric protein PML/RARA becomes a potential transcriptional repressor with altered DNA-binding properties and the capacity to block myeloid differentiation. The treatment of PML/RARA carriers with pharmacologic doses of retinoic acid in the form of ATRA destroys the co-repressor complex and leads to differentiation of the malignant promyelocytes (Guidez et al., 1998). At least four rare variant translocations associated with APL phenotype have been described: t(11;17) (q23;q12) ZBTB16/RARA, t(11;17)(q13;q12) NUMA1/RARA, t(5;17)(q35;q12) NPM1/RARA and t(17;17)(q11.2;q12) STAT5B/RARA. Some of these variants, the t(11;17) ZBTB16/RARA and t(17;17) STAT5B/RARA, are not sensitive to ATRA, so the molecular characterization of APL is necessary. The breakpoints in RARA gene are restricted to intron 2, while in PML gene there are two major breakpoints leading to long (L-form, bcr 1) or short (S-form, bcr3) transcript variant.

MLL gene on the chromosome 11q23 is an exceptional target in AML; as it is involved in at least 73 different translocations with more than 50 different partner genes (Huret, 2011) including different leukemia subtypes, such as de novo AML, therapy-related AML, myelodysplastic syndromes and acute lymphoblastic leukemia. The protein MLL is a histone methyltransferase that modulates gene expression, especially the expression of HOX genes, via chromatin remodeling (Dou & Hess, 2008), while most fused partners are supposed to be transcription factors. An exact mechanism of leukemogenesis involved different MLL translocations has not been completely understood. The t(9;11) (p22;q23) MLL/MLLT3 (MLL/AF9) is the most common, typically associated with subtype FAB-M5 having monocytic features. In contrast to most other MLL rearrangements that are associated with a poor prognosis, this translocation is associated with an intermediate prognosis. The breakpoints in MLL are clustered in a breakpoint cluster region, spanning exons 5–11. An extreme heterogeneity of translocations associated with MLL makes analysis using PCR diagnostic assay laborious. FISH is applicable for detection of MLL translocations in clinical practice (Keefe et al., 2010). Other significant MLL translocations in AML are t(6;11) MLL/MLLT4 (MLL/AF6), t(11;19) MLL/ELL, t(10;11) MLL/MLLT4 (MLL/AF10).

One of the rarer recurrent translocations the t(6;9)(p23;q34) occurs in approximately 1% of AML cases. The inv(3) and related t(3;3) translocation are found in approximately 1–2% of AML cases and associated with a poor clinical outcome (Swerdlow, 2008). AML with the t(1;22) translocation occurs primarily in infants representing less than 1% of all cases of AML. It is particularly associated with acute megakaryoblastic leukemia (FAB M7) and a good prognosis, if treated with intensive chemotherapy (Duchayne et al., 2003).

1.2.2 Gene mutations
Approximately 45% of AML cases have a normal karyotype but carry submicroscopic and cryptic genetic lesions that cannot be detected by conventional cytogenetics. Several acquired gene mutations have been described and characterized in AML. Two lesions (NPM1 and CEBPA mutations) have obtained provisional status in the 2008 WHO classification (Swerdlow, 2008).

NPM1 encodes a 37 kDa protein with versatile biologic activity that shuttles between the nucleolus, nucleoplasm and the cytoplasm (Grisendi et al., 2006; Meani & Alcalay, 2009). Mutations in NPM1 are common in AML, occurring with an overall frequency of 25–30% in adults and are associated with normal karyotype (Schnittger et al., 2005; Thiede et al., 2006). Most mutations in NPM1 are small insertions (4–11bp) in the terminal coding region (exon
12. Although over 50 mutations have been described, three specific mutations (A, B and D) account for the majority of the changes observed in AML (Falini et al., 2009). As a general rule, the presence of an \textit{NPM1} mutation is associated with a more favorable clinical outcome. However, the \textit{NPM1} mutations are often found together with an internal tandem duplication \textit{FLT3–ITD}, in this case the negative prognostic impact of a \textit{FLT3–ITD} mutation may override the positive value of an \textit{NPM1} mutation.

\textit{FLT3} is a class III receptor tyrosine kinase and a member of immunoglobulin receptor superfamily. It is expressed mostly in progenitor cells; the level of expression is lowered during differentiation. The ligand binding with \textit{FLT3} protein results in a phosphorylation of a juxtamembranous (JM) domain leading to the proliferation and inhibition of apoptosis. Two major types of lesions in \textit{FLT3} gene have been described: an internal tandem duplication (ITD) of the JM domain and a missense mutation D835mut in the activation loop (PM). Functionally, these lesions result in the constitutive activation of the tyrosine kinase domains via autophosphorylation and, consequently, to permanent signaling through downstream effectors. \textit{FLT3–ITD} is an independent predictor of poor clinical outcome (Meshinchi & Appelbaum, 2009). The prognostic value of the \textit{FLT3–PM} is controversial (Yamamoto et al., 2001). Another important diagnostic target is \textit{c-KIT} gene which encodes a type III tyrosine kinase involved in a signal transduction. The activating mutations, occurring in approximately 2–8% of all AML, lead to ligand independent signal transduction (Cairoli et al., 2006). In adults, the \textit{c-KIT} mutations typically predict worse prognosis, but it may be different in children (Pollard et al., 2010).

Due to significant progress in our understanding of acute leukemia pathogenesis, risk stratification of patients more and more relies on molecular genetic markers. New molecular targets will be found to enrich a clinician’s repertoire for making well-founded decisions regarding diagnosis, prognosis and therapy. This requires the establishment of appropriate and practical testing algorithms and development of standardized assays in order to obtain reliable and reproducible results.

1.3 Methods for detection of genetic lesions
Molecular diagnostics, first being a subsidiary tool in the clinical evaluation of acute leukemia, nowadays has been integrated rapidly into clinical laboratory practice. Genetic abnormalities associated with different leukemia subtypes can be detected by a variety of supplemental clinical methods, including conventional G-banded cytogenetics, FISH, PCR and DNA sequencing. The innovated technologies, such as gene-expression analysis, comparative genetic hybridization (CGH) and SNP arrays, still remaining at the experimental level, have a potential to hold a place in routine clinical evaluation and management of the disease. A brief overview of different methods allowing detection of different genetic lesions is presented in Table 1. Among them gel-based diagnostic biochips are considered, which are discussed further in more details.

A traditional karyotyping, based on Giemsa-stained metaphase chromosome spreads, continues to provide valuable information about translocations, as well as numerical changes in chromosome number and structure. Diagnostics of clonal process in a clinical sample is typically defined by the presence of at least two cells with the same structural abnormality, and at least three cells with the same abnormality in the case of a chromosomal loss. Despite limitations in analytic sensitivity and availability of dividing cells in a clinical sample, traditional karyotyping remains one of the most robust tools in molecular hematopathology.
Fluorescence in situ hybridization (FISH) involves fluorescently labeled probe binding to specific chromosome sequences that is visualized under fluorescent microscope. The structural microscopic or submicroscopic cryptic lesions, as well as different numeric chromosomal changes can be identified depending on a probe design (Wolff et al., 2007). FISH is more direct method to evaluate chromosomal lesions comparing with karyotyping, also it is more sensitive and can be applied to non-dividing interphase cells.

PCR is a popular and versatile tool in evaluation of acute leukemias. The technique is based on the exponential amplification of a target nucleic acid sequence; specific applications depend upon the type of initial nucleic acid and the detection method. The DNA-based allele-specific PCR is used for the detection of gene mutations. The RNA-based PCR assay, reverse-transcription PCR or RT-PCR, is extremely useful in the analysis of fusion genes which express chimeric transcripts. Also the measurement of gene expression is possible. The resulting amplicons are evaluated either quantitatively by a real-time PCR approach or qualitatively by gel electrophoresis after 30-35 cycles of amplification. Because of the exponential amplification of target, sensitivity of the method is very high allowing detection of 1 blast with specific lesion among $10^{-4}-10^{-5}$ normal cells (Rennert et al., 1999). Because of its increased analytical sensitivity regarding to karyotyping and FISH, RT-PCR is widely used for monitoring minimal residual disease in leukemia cases with recurrent translocations.

<table>
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<th>Parameters</th>
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Table 1. Characteristics of different molecular genetic methods in the analysis of acute leukemias

The variability and complexity of mutations in some genes, like NPM1 or CEBPA, make them ineligible for standard PCR assays, in such cases direct DNA sequencing can be used to search for the presence of a genetic lesion (Ahn et al., 2009). The traditional sequencing is a robust and flexible methodology that reliably detects the presence of mutations when approximately 20% of cells carry the genetic lesion. While not available clinically, next-generation DNA sequencing methods become more and more applicable regarding to time, money and data analysis. In 2010, it is feasible to sequence a human genome in

1 - except cryptic aberrations;
2 - the MRD level is 0.001% for translocations and 5% per gene mutations

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approximately 1 week for approximately US$15,000 (Aparicio & Huntsman, 2010). Different microarray-based methodologies are applied to profiling gene expression and measuring copy number variations (CNV) (Bacher al., 2010; Nasedkina et al., 2009). It has been shown that the gene expression measurement is independently capable of classifying many of the clinically relevant ALL and AML categories based on their distinct expression profiles (Bullinger et al., 2004; Radmacher et al., 2006). At the same time, expression arrays can not predict the mutation status of several prognostically relevant genes, such as FLT3 (Kohlmann et al., 2010). Further standardization of these complex techniques is needed for successful introduction into clinical laboratories (Haferlach et al., 2010).

2. Diagnostics of molecular markers in childhood leukemia using biochips

The translation of research to practice has been stimulated the development of novel approaches to clinical diagnostics potentially capable to overcome the limitations of settled technologies, like karyotyping, FISH and RT-PCR. Complicated assays using high-density arrays hardly are used in daily routine diagnostics, especially in small clinical laboratories. To overlap this gap different low-density array-based techniques have been developed, which are less complex, less expensive and more reproducible systems with a few molecular markers comparing to high-density microarrays. Gel-based biochips are a good example of such a technology.

2.1 Gel-based biochip technology

Overviews of low-density gel-based biochip or hydrogel biochip technology and applications in different fields exemplifying diagnostics from infectious diseases to protein oncological markers have been published recently (Mikhailovich et al., 2008; Nasedkina et al., 2009; Rubina et al., 2008). Principal difference of gel-based biochip technology comparing with other matrix microarrays is an immobilization of identifying probes in semi-spherical three-dimensional (3-D) gel elements instead of the flat supporting surface. The solution containing oligonucleotide probes mixed with gel-forming monomer is placed on a activated plastic or glace surface by a standard robotic device. Oligonucleotide probes are modified and carry NH₂-groups at their 3’ ends. Copolymerization of molecular probes and main gel components is processed under UV-light. As a result the immobilized molecules become covalently bound to monomers of a growing polymer chain and distribute evenly throughout each gel element, as shown by confocal microscopy (Rubina et al., 2004). The diameters of gel elements are ranged from 50 up to 300 µm with a distance between them from 100 up to 500 µm depending on experimental tasks. A number of gel elements vary from several tens up to several thousands depending on the complexity of target analyzed. The quality control of gel element disposition is performed using specialized optical device and computerized image analysis. Such quality control allows minimizing intra- and inter-array variation of a drop size on ready-to-use biochips and substantially increases the reproducibility of hybridization results with different series of biochips. Fluorescent dyes are used as labels to register hybridization pattern. Different analogs of cyanine fluorescent dyes Cy3 and Cy5 have been synthesized to increase the sensitivity and efficacy of hybridization analysis (Kuznetsova et al., 2008). In the case of DNA sequence analysis the hybridization target represents a fragment of genome and the amplification of DNA is usually needed using PCR with simultaneous incorporation of fluorescent label. The size of amplified fragments is in range of 100-1000 b.p. For proteome analysis gel elements contain antigen or antibody and the fluorescent label is conjugated directly with target
analyzed or with molecules of developing antibodies like in the sandwich immunoassay. A biochip photograph and view under light microscope are presented in Fig.1, A and B.

A – General view of biochip with hybridization chamber;
B – gel elements with a diameter of 100 µm under light microscope;
C – portable biochip analyzer (on the right). Scale bar = 200 µm.

Fig. 1. Gel-based biochips.

The higher probe concentration in gel drops compared with spots of 2-dimensional microarrays allows the use of a simple detection system consisting of a laser source equipped with lenses and charge-coupled device camera (Fig. 1, C). The portable biochip analyzer is certified for clinical use by a national regulatory agency, the Ministry of Public Health of the Russian Federation (Registration Certificate of Federal Service for Supervision in Public Health Sphere No. FS 022а2006/3777-06). Image analysis is performed using automated user-friendly software «Imageware®», which measures the fluorescence intensities in gel elements after allele-specific hybridization and determines the gel elements where the formation of perfect duplexes is performed between complementary probe and target. Then the system presents a report about the presence of mutation, polymorphism or chromosome translocation in a sample under analysis.

The main features of the gel-based biochip analysis are its simplicity, and the low cost of equipment and biochips themselves (approximately 8 $US per chip in 2011), enabling their use by small diagnostic laboratories. One of the first clinical biochip applications was the analysis of chromosomal translocations occurring in leukemia.

2.2 Analysis of chromosomal translocations in leukemia

The recurrent chromosomal aberrations represent important diagnostic and prognostic markers of different types of disease requiring appropriate therapy and are taken into
account in the risk-stratification of patients in different clinical trials (Look, 1997; Möricke et al., 2008; Rubnitz et al., 2008; Rabbits, 1994; Shrappe, 2004). When an aberration is found, it also serves as a reliable target for monitoring of minimal residual disease. Traditionally, chromosomal translocations have been identified using cytogenetics and FISH. However, the analysis of chromosomal translocations with cytogenetic methods is not always accurate and representative, because of submicroscopic lesions and cryptic translocations. The FISH technique is comprehensive, reliable and available for clinics approach, but it requires expensive basic equipment, does not allow the detection of all targets of interest simultaneously and is not sensitive enough for effective monitoring of MRD. The reverse-transcription (RT)-PCR is another widely used method able to detect chimeric transcripts, which derive from fusion genes and represent an excellent molecular target expressed in leukemic blasts (Braziel et al., 2003; Haferlach et al., 2005). As far as the identification of each transcript requires an individual PCR reaction with specific primers, a scale of the study increases markedly when each patient is analyzed for several transcripts simultaneously. Thus, multiplex protocols have been developed to diminish time- and labor-intensity of the procedure (Pallisgaard et al., 1998; Scurto et al., 1998). The multiplex RT-PCR assay is usually multi-stage: the multiplex reaction is followed by series of identifying PCR reactions with primers specific for individual translocations. To facilitate the identification of recurrent translocations an oligonucleotide biochip has been developed and combined with multiplex RT-PCR assay. The hybridization step increases specificity of the assay while reduces associated costs and amount of patient material required. Taking into consideration the results of clinical trials, thirteen chromosomal aberrations considered the most important for diagnosis and prognosis, were chosen as targets (Mitiaeva et al., 2004; Nasedkina et al., 2003). The following translocations were included into the assay: t(9;22)p190 and p210, t(12;21), t(1;19), t(8;21), inv(16), t(4;11), t(9;11); t(10;11), t(11;19)ELL, t(11;19)ENL. Oligonucleotide probes of 20–25 bp in length were spotted in duplicates in order to increase reproducibility of hybridization results. The biochip contains probes for the detection of wild type ABL gene, which is expressed in all cells. The detection of ABL transcript is used to control the quantity and quality of isolated RNA and to estimate the effectiveness of labeled target preparation. For each translocation, a set of probes was designed including a so-called common probe from one of the genes involved in the translocation, and also specific probes to identify the breakpoint variants. The procedure includes the isolation of RNA from bone marrow or peripheral blood cells, multiplex RT-PCR using primers specific for different translocations, labeling of single strand PCR product via incorporation of modified Cy5-dUTP in the course of asymmetric PCR, hybridization on the biochip, and registration of fluorescence intensities in gel elements of biochip using a biochip analyzer. Computer-based processing of fluorescent signals using a specially developed algorithm allows an automated image analysis. Examples of different hybridization patterns are presented in Fig. 2, A and B. The limit of sensitivity is one blast cell with a chromosomal translocation among 10³–10⁴ normal cells.

The MLL translocations represent a difficult subject for analysis by conventional cytogenetics or standard RT-PCR due to high diversity of gene partners involved in translocations with MLL gene. In MLL translocations the fusion results in a gene and transcript with the 5’ end belonging to MLL gene originally located in 11q23 region and the 3’ end belonging to one of more than 50 partner genes (Eguchi et al., 2005). In biochip-based assay a set of probes specific for different MLL exons and probes specific to different exons
Fig. 2. Identification of chromosomal translocations by Leukogen-biochip. Two lower spots represent fluorescent marker, two upper spots correspond to ABL-gene specific signals. A – a sample of patient carrying no translocations; B- a sample of patient carrying translocation t(15;17), fusion gene PML/RARA, bcr3 breakpoint variant.

of partner gene is used. The analysis was done as described previously (Mitiaeva et al., 2004). The biochip-based approach can indicate which MLL exon is involved in formation of a chimeric transcript, as well as identifies the partner gene. Most frequent translocations in ALL and AML are included into assay, namely t(4;11), t(6;11), t(9;11); t(10;11), t(11;19)ELL, t(11,19)ENL. They are included in the last version of Leukogen-biochip for the analysis of 13 translocations. Another diagnostic biochip has been described which allowed the analysis of about thirty different gene partners, but the sensitivity limit of the assay is significantly lower: 1 blast cell per 10 normal (Maroc et al., 2004).

During 2006-2009 the bone marrow samples of patients with leukemia of age 0 to 18 treated in hematological clinics were received and tested using the biochip-based approach for the presence of translocations. The hematologic malignancies ALL and AML were diagnosed according to standard criteria by cytomorphologic, cytochemical, and immunophenotypic studies of bone marrow cells. Totally, 1200 children with leukemia from different parts of Russia were included into assay: 912 patients were diagnosed with ALL and 282 with AML. In ALL patients the chromosomal aberrations analyzed were found in 24.4% of all cases. In
AML the portion of patients carrying specific translocations was 36.4% of all cases. The most frequent translocation in ALL was cryptic translocation t(12;21) (12.8%), fusion transcript TEL/AML1 or ETV6/RUNX1. In AML most frequent translocations are t(8;21) (9.5%) with fusion gene AML/ETO or RUNX1/RUNX1T1; t(15;17) (8.0%) with fusion gene PML/RARA and t(9;11) (10.5 %) with MLL/MLLT3. The frequency of t(12;21) obtained in Russia is lower than the frequencies of 18-20% found in other countries (Hilden et al., 2006; Rubnitz et al., 2008; Vilmer et al., 2000).

Validation of the method was done using individual RT-PCR protocols for each translocation (Pallisgaard et al., 1998), in others words, for 325 patients the standard RT-PCR assay was performed parallel to biochip-based analysis. The concordance between the two methods was about 98%. The main source of false-positive signals on biochip may be contamination by amplified DNA fragments; the problem can be overcome by more accurate PCR handling. To avoid false-negative results, biochips were tested periodically with control samples carrying translocations. The results of the biochip analysis were also compared with clinical data and a morphologically determined leukemia variant. The patients carrying translocations were further monitored for minimal residual disease to follow efficacy of therapy and to predict a relapse. The Leukogen-biochip was certified by a national regulatory agency, the Ministry of Public Health of the Russian Federation, for clinical application (Registration Certificate of Federal Service for Supervision in Public Health Sphere No. FS 012b2006/4756-06).

2.3 Analysis of gene mutations using biochip

Gene mutation analysis using gel-based biochips included following steps: DNA isolation from biological sample, two-round multiplex PCR of gene fragments containing the mutations analyzed, labeling of PCR products with Cy-5-dUTP during an asymmetric PCR of the second round, hybridization on biochip, registration of fluorescent signals and image analysis of hybridization patterns. The hybridization with allele-specific probes allows distinguishing between wild-type and mutant alleles with high fidelity (Nasedkina et al., 2006). Mutations in NPM1 gene are considered as genetic markers defining patient’s subgroups with different clinical characteristics. The genetic lesions in NPM1 gene are mostly presented by tetra nucleotide insertions in exon 12. A biochip has been developed for the analysis of about 20 different insertion types in NPM1 gene. The biochip for the analysis of NPM1 gene mutations and hybridization patterns is presented in Fig. 3. A clinical screening was performed to identify the NPM1 mutations in 188 de novo diagnosed pediatric AML cases previously investigated for the presence of aberrations with Leukogen-biochip. The NPM1 mutations appeared only in patients without chromosomal aberrations and this association with normal karyotype was statistically significant (p<0.05). Frequency of mutations was 4.8% (9/186) of total AML patients group and 8.0% (9/112) of patients without aberrations. The mutations in NPM1 gene were represented by 4 b.p. insertions: TCTG (type A), CATG (type B), CCTG (type D), TCGG (type Qm). The nomenclature of mutation types is given as described previously (Rau & Brown, 2009). All patients with mutations were heterozygote carrying one mutant and one wild-type alleles. In adults, the mutations in NPM1 gene are considered most frequent events in AML, especially in AML with normal karyotype. The mutation frequency is ranged between 12%-18% (Falini et al., 2005; Thiede et al., 2006; Rau & Brown, 2009). In childhood AML it seems to be relatively rare occurring in 2-7% of all cases (Brown et al., 2007; Cazzaniga et al., 2005;
Fig. 3. Analysis of the mutations in NPM1 gene. A - scheme of biochip, B – hybridization pattern for patient homozygous for wild-type NPM1 allele; C – hybridization pattern for patient carrying mutation type B, wild-type allele is also presented (WT – wildtype, A-Qm – mutation types).

Renneville et al., 2008). In our study the NPM1 mutations were found in 4.8% of all patients and in 8.0% of patients without chromosomal aberrations, thus the data corresponded to those previously described. As far as NPM1 mutations occur only in patients with normal karyotype it is possible to use them as potential diagnostic markers for MRD monitoring. The high diversity and complex nature of the NPM1 mutations make difficult the analysis using PCR methods, more often direct sequencing is applied. In this case the biochip-based hybridization analysis may be a method of choice allowing detection of a wide spectrum of NPM1 insertions. The usefulness of biochip-based approach becomes apparent in analysis of many mutations in different genes simultaneously. Further development of NPM1-biochip is suggested to include most important mutations in FLT3 and c-KIT genes. The parallel usage of two kinds of diagnostic biochips, one for translocations and another for mutations may significantly improve molecular diagnostics of leukemia.

2.4 Pharmacogenetic testing and personalized treatment
Germline polymorphisms in genes, which hypothetically can mediate differential responses to drugs in leukemic patients, are perspective targets for investigation. Such genes are involved in drug absorption and excretion, metabolism, cellular transport and the drug
targets and pathways of standard chemotherapeutics. Polymorphisms in these genes can potentially lead to higher or lower levels of chemotherapeutic drug response and consequently might affect toxicity or efficacy. Numerous studies have investigated an association of polymorphisms in different genes coding drug-metabolizing enzymes with patient responses to chemotherapy. The cytochrome P450 enzymes are involved in the Phase I metabolism of many antileukemic agents including cyclophosphamide, etoposide, doxorubicin and vincristine, and might influence on ALL therapy outcomes (Fleury et al., 2004; Rocha et al., 2005). A number of Phase II metabolism enzymes are involved in inactivation of antileukemic agents. Through glutathione conjugation, glutathione S-transferases (GSTs) generally inactivate glucocorticoids, vincristine, anthracyclines, cyclophosphamide and epipodophyllotoxins. Polymorphisms in various GST genes (GSTT1, GSTM1, GSTP1) have been extensively studied in prognosis of childhood ALL (Anderer et al., 2000; Davies et al., 2002; Kishi et al., 2004; Stanulla et al., 2000).

2.4.1 Biochip for the analysis of drug-metabolizing genes
To investigate association between polymorphisms in drug-metabolizing genes and response to therapy and clinical outcome a Pharmagen-biochip has been designed. The following targets were included into assay: genes CYP1A1, CYP2D6, CYP2C9, CYP2C19, coding cytochromes, enzymes of Phase I of biotransformation; genes GSTT1 and GSTM1, coding glutathione S-transferases (GST), gene NAT2, coding N-arylamino acetyl transferase, gene TPMT, coding thiopurine-S-methyl transferase, which products are involved in different Phase II reactions; and also MTHFR gene, which product participates in folate metabolism. An example of sample analysis using Pharmagen-biochip is given on Fig. 4. Finally, the Pharmagen-biochip allows analyzing 16 SNP’s and 2 deletions in 10 genes CYP1A1 (4887C>A, 4889A>G, 6235T>C), CYP2D6 (1934G>A, 2637delA), NAT2 (481C>T, 590G>A, 857G>A), MTHFR (677C>T), CYP2C9 (430C>T, 1075C>T), CYP2C19 (681G>A), , TPMT (238G>C, 460G>A, 719 A>G), GSTT1 (deletion) and GSTM1 (deletion). Accuracy of the analysis was found to be no less than 98% in experiments with control samples of known genotype. The Pharmagen-biochip was approved by the Ministry of Public Health of the Russian Federation for clinical testing of drug-metabolizing enzyme polymorphisms (Registration Certificate No. FS 012b2006/5317-06)

2.4.2 Polymorphism of thiopurine-S-methyltransferase 6-MP toxicity
One of the well known antileukemic drugs is 6-mercaptopurine (6-MP), which is used for supportive therapy in ALL. Within the cell thiopurines are metabolized into active thioguanine nucleotides (TGNs) which incorporate into DNA or RNA and result in cell cycle arrest and apoptosis. Like other thiopurine drugs, 6-MP is metabolized mainly by a highly polymorphic enzyme thiopurine-S-methyltransferase (TPMT) (Evans & McLeod, 2003). The relationship between TPMT polymorphisms and thiopurines efficacy and toxicity in children with ALL is a paradigm of the clinical application of pharmacogenetics (Evans & McLeod, 2003; Stanulla et al., 2005; Relling et al., 1999). The TPMT activity is inherited in an autosomal dominant manner and demonstrates genetic polymorphism: about 91% of people have polymorphic variants with high activity, an intermediate TPMT activity occurs in about 9% of the population and 0.3% have low or undetectable TPMT enzyme activity (Yates et al., 1997). The wild-type allele, TPMT*1, encodes an active TPMT enzyme. While
Fig. 4. Image analysis of hybridization pattern. For correct genotype assignment the average signals from two upper and two lower drops in each column are divided on the strongest signal and normalized signals are compared (see diagram on the right). Two threshold lines are used to distinguish between homozygotes and heterozygotes. For homozygotes one signal from wild-type or mutant allele is maximal and another should not exceed the lower threshold. The discrimination between positive and negative signals is more than 5 fold. For heterozygotes both signals from wild-type and mutant alleles should not descend below the upper threshold.

Many variant alleles of TPMT have been identified, three account for more than 95% of inherited TPMT deficiency: $TPMT^{*2}$ (238G>C), $TPMT^{*3A}$ (460G>A, 719A>G) and $TPMT^{*3C}$ (719A>G) (Krynetski & Evans, 2003). It was demonstrated that specific TPMT variants have low activity due to aggregation of variant proteins, providing a structural explanation for the observed differences in TPMT activity (Wang et al., 2005). The patients with very low or undetectable TPMT activity are at high risk of severe, potentially fatal hematopoietic toxicity when they are treated with standard doses of thiopurines (Evans et al., 1991). At the same time they also have relatively lower levels of residual leukemic blasts and may be at lower risk of relapse (Lennard et al., 1990). The TPMT heterozygotes display a phenotype intermediate between the two homozygous states (Evans et al., 2001). Because of these data, the prospective testing of $TPMT$ gene status in ALL patients is recommended to allow preventive dosage reductions in those with low enzyme activity to minimize the treatment toxicity. In some trials patients heterozygous for TPMT-deficient alleles received reduced 6MP dose, but the risk of relapse is not higher than for those with wild type TPMT, who were treated with full dose (Relling et al., 2006; Evans et al., 1998).

The most frequent $TPMT$ gene mutations leading to enzyme deficiency in Russian population were analyzed using biochips (Nasedkina et al., 2006). The genotyping of
patients included DNA isolation from peripheral blood leukocytes, multiplex PCR, hybridization with the biochip, and image analysis. A total of 446 children with hematologic malignancies were genotyped using the TPMT-biochip (Samochatova et al., 2009). Of 241 patients with ALL for whom molecular analysis has been performed, 18 patients (7.5%) were heterozygous carriers of polymorphic alleles with deficient enzyme activity. The most frequent deficient allele was TPMT*3A (2.3%), while more rare alleles were TPMT*3C (0.4%) and TPMT*2 (0.1%); the wild-type allele TPMT*1 accounted for 97.2%. Thus, spectrum of TPMT deficient alleles and their frequencies in Russian children with leukemia was close to those in European populations and in white population of USA. A retrospective analysis of the 6-MP therapy intolerance stratified by TPMT genotype was carried out using the TPMT-biochip. Dosages were adjusted to keep the WBC count between 2 and 3x10⁹/Lm, but not on the basis of TPMT genotype. Eighteen ALL patients heterozygous for TPMT deficient allele were characterized by decreased ability to tolerate the 6-MP therapy, and as a result they received significantly lower doses of this drug compared with patients with a homozygous wild-type genotype (average weekly dose of 6-MP was 264 vs 312 mg/m² respectively; p = 0.04). Also they received more erythrocyte and thrombocyte transfusions and had more infectious episodes. Despite all deviations from protocol, the results of treatment did not differ significantly between patients who did and did not have the TPMT gene variations. An EFS estimate was 90% for patients with variations and 83% for patients with wild-type genotype at a median follow-up of 31.3 months (p= 0.562). It may be explained by a fact that the treatment levels of intracellular TGN (the active metabolite of 6MP) were achieved in both cases, regardless TPMT genotype. Thus, TPMT genotyping can be highly recommended to those patients treated with 6MP, who is found to have repeated episodes of prolonged cytopenia, to adjust an individual drug dosage (Samochatova et al., 2009).

2.4.3 Polymorphism of drug-metabolizing enzyme genes and risk of relapse in ALL

Other different polymorphic enzyme variants that have decreased or increased activity can potentially modulate clinical response to anticancer therapy. This may lead to increased toxicity of the treatment, but also to the development of drug resistance or increased risk of relapse or secondary tumor development (Balta et al., 2003; Krajinovic et al., 2002; Rocha et al., 2005). The most important enzymes of phase I biotransformation enzymes, cytochromes of the P450 family (CYP1A1, CYP2D6, CYP2C9, and CYP2C19), which activate xenobiotics to yield genotoxic intermediates, and phase II enzymes, such as glutathione S-transferases (GSTs) and arylamine N-acetyltransferases (NATs), which convert genotoxic compounds to nontoxic compounds, were chosen for the analysis. Using Pharmagen-biochip the frequencies of the polymorphic variants of CYP1A1, CYP2D6, GSTT1, GSTM1, MTHFR, CYP2C9, CYP2C19, and NAT2 in 332 children with acute lymphoblastic leukemia (ALL) were determined. Among them, 258 patients with primary leukemia, having no relapse within 1 year of therapy, and 74 with relapse were included in the study. Our results demonstrated that some allelic variants of the drug-metabolizing genes were associated with a higher risk of relapse in childhood acute leukemia (Fig. 5). The association of polymorphic variant CYP1A1*2A with poor therapeutic prognosis has been shown in children with ALL (OR = 2.3, 95% CI = 1.09 - 4.8, p = 0.03) (Gra et al., 2009). Also it was found that the GSTT1 null genotype occurred rarely in children with relapse as compared with those with one or two functional alleles (OR = 0.48, 95% CI = 0.26 – 0.90, p = 0.02), thus being protective. The presence of both risk genotypes has an additive effect: the
frequency of relapse was higher among carriers of the combined CYP1A1 *1/*2A and GSTT1 non-null genotype (OR = 2.36, CI = 1.02 – 5.46, p = 0.048).

![Genotype Frequencies in Primary Leukemia and Relapsed Patients](image)

Fig. 5. The genotype frequencies in primary leukemia and relapsed patients with pediatric ALL (statistically significant difference is marked by asterisk).

The observed regularities corresponded to published data (Anderer et al., 2000; Stanulla et al., 2000; Voso et al., 2005). An increase in frequency of the CYP1A1 genotype *1/*2A in children with ALL relapse may be explained by the impact of synthetic glucocorticoids (such as dexametasone and prednisolone) being an important component of the ALL treatment protocol and induction therapy. They cause lymphopenia and involution of lymphoid tissue that lead to immunosuppression. The increased enzymatic activity of the CYP1A1 *1/*2A leads to the increased concentration of intermediate genotoxic metabolites and of total mutagenic activity (Voso et al., 2005). Since the formation of additional mutations may cause resistance of cancer cells towards therapy, it is likely that the CYP1A1 *1/*2A genotype may decrease the efficacy of therapy and promote the development of relapse. GSTs are involved in metabolism of many antitumor drugs, catalyzing conjugation of intermediate metabolites with reduced glutathione. The ALL children carrying GSTT1 null genotype have been observed to respond well to induction therapy with prednisone, while carriers of at least one functional GSTT1 allele displayed glucocorticoid resistance, a poor response to therapy, and a higher relapse rate (Anderer et al., 2000). It is likely, that in carriers of the GSTT1 and/or GSTM1 null genotype, lack of GSTT1 and/or GSTM1 enzymes leads to an accumulation of cytotoxic drugs that may enhance their efficacy and longer relapse-free survival.

3. Conclusion

The modern diagnostics of leukemia is multifaceted, including clinical characterization, histochemistry, cell morphology, immunophenotyping and also molecular genetic analysis. Molecular markers, recurrent chromosome aberrations and gene mutations, allow subdividing leukemia patients into biological groups with unique clinical features. The risk-stratification of patients based on genetics of leukemia blasts contributes not only to classification of leukemia subtypes, but can predict prognosis and clinical outcome. The molecular genetic analysis provides clinicians with an important knowledge for a decision
making and a choice of appropriate therapy. Further progress in therapy of oncologic
diseases is inseparable from individualization of the treatment based on the molecular
characteristics of malignant cells and genetic features of a patient. An introduction of new
technologies in routine clinical practice can significantly increase the power of modern
diagnostics. Low-density gel-based biochips are a good example of such a technology. The
diagnostic biochips may provide easily, rapid genotyping in clinics and be a useful tool in
large screening programs. The properties of gel-based biochips are defined by
immobilization in 3D-volume of hydrogel: high concentration of probe due to
immobilization capacity of gel and high level of fluorescent signal enabling usage of a
simple detecting device, like a portable biochip analyzer. Although hybridization on 2D-
microarrays is slightly faster, gel-based biochips provide better discrimination between
perfect and imperfect duplexes (5- to 20-fold difference). Thus, unambiguous interpretation
of results is attained that can be crucial for clinical diagnostics. Different genetic
abnormalities may be identified successfully with diagnostic biochips: from balanced
translocations forming fusion genes to gene mutations or polymorphic variants.

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Diagnostics of Molecular Markers in Childhood Acute Leukaemia Using Biochips


This book provides a comprehensive overview of the basic mechanisms underlying areas of acute leukemia, current advances, and future directions in management of this disease. The first section discusses the classification of acute leukemia, taking into account diagnoses dependent on techniques that are essential, and thankfully readily available, in the laboratory. The second section concerns recent advances in molecular biology, markers, receptors, and signaling molecules responsible for disease progression, diagnostics based on biochips and other molecular genetic analysis. These advances provide clinicians with important understanding and improved decision making towards the most suitable therapy for acute leukemia. Biochemical, structural, and genetic studies may bring a new era of epigenetic based drugs along with additional molecular targets that will form the basis for novel treatment strategies. Later in the book, pediatric acute leukemia is covered, emphasizing that children are not small adults when it comes to drug development. The last section is a collection of chapters about treatment, as chemotherapy-induced toxicity is still a significant clinical concern. The present challenge lies in reducing the frequency and seriousness of adverse effects while maintaining efficacy and avoiding over-treatment of patients.

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