Chapter

Latest Implications of Next-Gen Sequencing in Diagnosis of Acute and Chronic Myeloid Leukemia

Oana Maria Boldura, Cristina Petrine, Alin Mihu and Cornel Balta

Abstract

The spectacular progress which was present in the past few years in the field of genome sequencing, together with the appearance on the market of some high performance devices in this field, the reduction of the costs regarding the analysis of the samples and the standardization of some protocols, has led to the establishment and introduction of the new generation of sequencing techniques in clinical diagnostic labs. An important role is played by the implementation of this technique in the oncology clinics. In this context, we found it appropriate to discuss in this chapter about the role of next-gen sequencing in determining the genetic probabilities of occurrence of oncological pathologies in the healthy population, the screening of these diseases at the population level, the diagnosis and classification of this pathology, the establishment of the therapeutic conduct using the technique, as well as the progression of the disease. In this chapter, we intend to discuss in particular the involvement of this technology in hemato-oncological diseases.

Keywords: next-generation sequencing, acute myeloid leukemia, chronic myeloid leukemia, molecular diagnosis

1. Introduction

The rapid development of the new sequencing techniques, the development of databases for the analysis and comparison of the various pathologies, as well as the reduction of the costs related to their exploitation and the total value of the cost related to the analysis, will lead to the implementation of this technique in the clinical diagnostic laboratories. Also the implementation of this technique leads to the need to develop and comply with standards, which will result in the development of valid and useful results to the clinician. This technique is used especially for the diagnosis and monitorization of hereditary diseases, being able to evaluate the genetic changes that appeared in both germline and somatic cell lines, focusing on evaluating mutations occurring at the level of a single gene, evaluating gene panels involved in the molecular pathobiology of various disorders, as well as the evaluation of the various protein-coding genes involved in this process [1]. In view of the above, we can conclude that this technique brings important information to the clinician, both about the presence of a possible mutation which will cause an affection, as well as about the metabolic interactions and the play of gene expression involved

in the pathobiological mechanism of diseases, thus offering not only an early and accurate diagnosis but also the possibility of highlighting molecular targets for therapy, as well as a precise assessment of the progression of the disorders, being necessary correlated with standardized clinical and paraclinical examinations.

The Food and Drug Administration (FDA) has prepared and finalized a document guiding the use of next-generation sequencing in germline disease assessment, which was published on April 12, 2018, under the name of "Considerations for Design, Development and Analytical Validation of Next Generation Sequencing (NGS) - In Vitro Based Diagnostics (IVDs) Intended to Aid in the Diagnosis of Suspected Germline Diseases," thereby trying to take a step forward to standardize and introduce this technique into the current practice of diagnostic laboratories, but at the same time making sure that the patient's safety is the number one priority in front of technological innovations and possible analytical errors [2].

Lately, the progress in this field has had as a direct consequence on the drastic decrease of the cost with this analysis, at the same time developing over 55,000 genetic tests for more than 11,000 pathological conditions.

Remarkable results could be noticed after the implementation of this technology in the diagnosis and follow-up of the progression of oncological pathologies, among them being noted Hodgkin's lymphoma, breast cancer, and chronic myelogenous leukemia. Also this technique brings real benefits in the diagnosis, understanding, and study of the progression of cardiovascular diseases in direct correlation with the therapy administered to digestive, respiratory, and nervous disorders. Particular importance must also be given to the power of this new technology to aid microbiological diagnosis, as well as its usefulness in establishing the resistance of pathogenic microorganisms to various anti-infectious agents.

2. Acute myeloid leukemia: patho-molecular mechanism and diagnosis

The introduction of the new generation sequencing techniques has led to the development of knowledge of the mechanisms that govern the gene mechanisms that trigger and lead to the progression of malignant oncological diseases of the myeloid line. Besides the chromosomal mutations revealed by classical cytogenetic methods, observed on a larger scale, the next-generation sequencing revealed numerous other genetic alterations, which could not be revealed using classical methods. These studies have revealed some genetic similarities in various morphologically distinct conditions, suggesting that they have a similar molecular mechanism, these mechanisms being represented by cell signaling, transcription, regulation of the cell cycle, regulation of DNA methylation, changes occurred in histone regulation, RNA splicing, and alterations of the components of the sister chromatid cohesion complex [3]. All these genetic alterations can represent a starting point in the development of molecular biomarkers, which can be easily monitored by the new sequencing techniques; in **Figure 1**, the complex genetic substrate, involved in the induction and evolution of the malignant pathologies of the myeloid line is presented, hence being instrumental in establishing the diagnosis, prognosis, and therapeutic option, some of them being already validated and used in current practice.

Acute myeloid leukemia is part of the myeloid hemato-oncological disorders with high aggression, which affects the blood cells, being the leukemia with the highest weight in the adult population, having an unfavorable prognosis, despite the spectacular progression of the new therapies applied to this pathology, which leads to the in-depth study and a better understanding of the molecular processes that occur during the evolution of the disease.

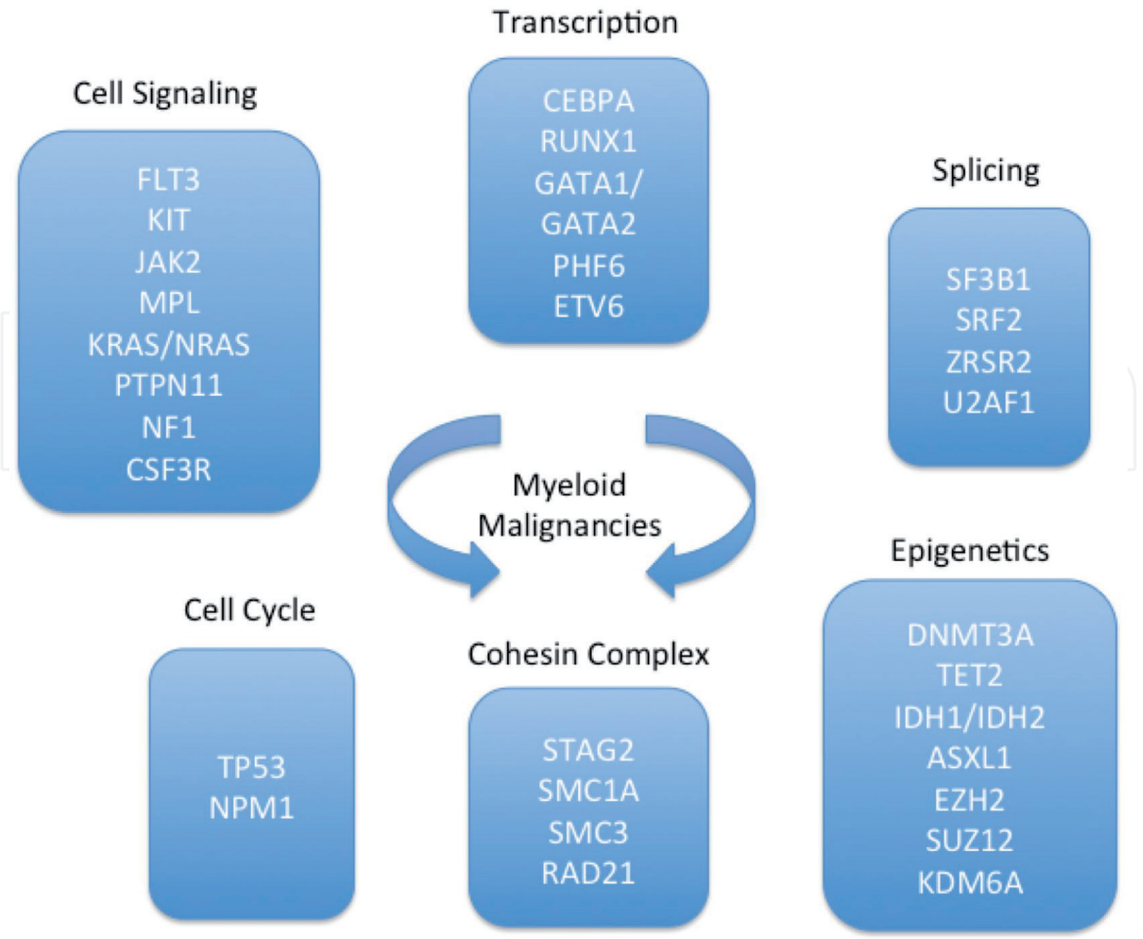

Figure 1.
Metabolic pathways affected in the oncological malignant pathology of the myeloid lineage that could be used as potential biomarkers in the diagnosis of these disorders [3].

An important first step in understanding the molecular and genetic mechanisms that governed the occurrence and progression of AML was the introduction of chromosome analysis by banding; this analysis provides relationships on the chromosome level changes, with direct resonance in the molecular pattern modification and clinically being specific for the disease. The first genetic alteration discovered and correlated with the evolution of promyelocytic leukemia was the translocation of t (15;17), but in the progression of this pathology, other chromosomal alterations appear, such as the translocation of t (8;21), inversion of 16, all of which are associated with a favorable prognosis, while the association of these alterations together with the existence of structural alterations will lead to the establishment of an unfavorable prognosis.

Mutations affecting the cell lines involved in AML can be classified into two main categories: Class I mutations leading to the promotion of monoclonal cell proliferation and Class II mutations leading to the inhibition of myeloid differentiation into mature, immunocompetent cell stages; this classification is illustrated in **Figure 2** [4].

Highlighting these mutations will lead to the diagnosis of most acute myeloid leukemias with normal cytogenetic profile. In this context, the new generation sequencing is a useful element in the discovery of leukemias with normal cytogenetic profile, being able to discover even new mutations involved in the progression of this pathology. Also in the pathogenesis of these diseases, not only the DNA substrate modifications are involved, represented by the gene mutations or chromosomal translocations, but also epigenetic mechanisms that dictate the expression

of these genes, such as the changes produced at the histone level and DNA methylation, may also be implicated. There are also miRNAs that can act as oncogenes or as tumor suppressor genes [5, 6]. Thus, the combination of new generation sequencing techniques, functional genomics and proteomics, will contribute to a better understanding, highlighting new therapeutic targets and new treatment modalities for AML in the future.

The alterations produced in the genetic material of the myeloid cell lines involved in the appearance of AML, lead directly to both functional and numerical alterations of these cells, as well as to structural and morphological alterations, alterations that are important in the primary hematological diagnosis, highlighted by smears made from peripheral blood samples or samples from the hematogenous spinal cord (**Figure 3**).

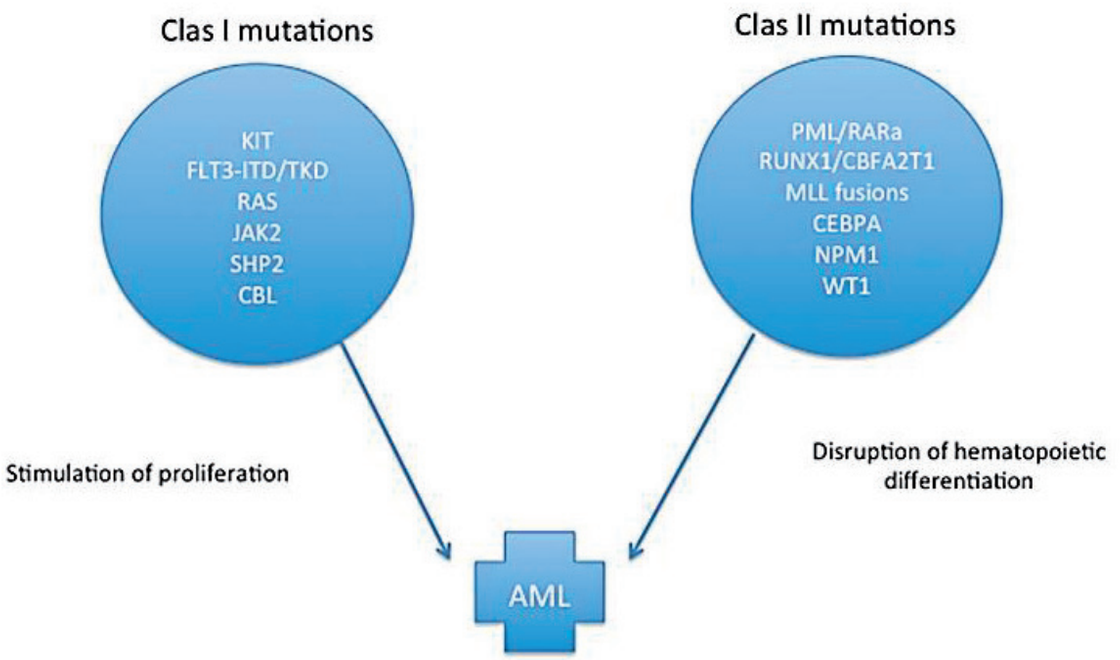

Figure 2.
Molecular models of mechanism involved in acute myeloid leukemia [4].

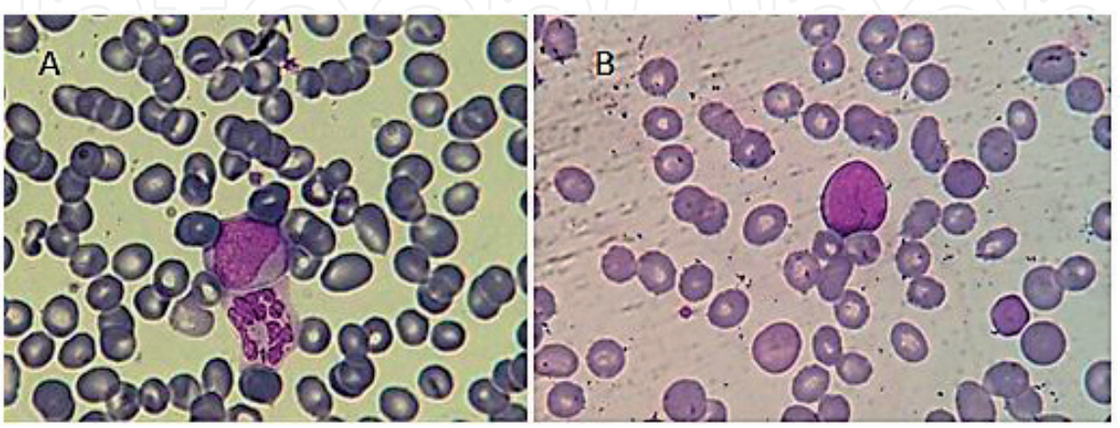

Figure 3.
Peripheral blood samples from a patient with AML, May Grunwald Giemsa stain (MGG), magnification stage 1000X. Picture (A) contains a myeloblast next to a dysplastic agranular hypersegmented neutrophil and picture (B) shows a myeloblast surrounding RBC that shows discrete anisochromia, slight anisocytosis, and a polychromatophilic RBC on the bottom right.

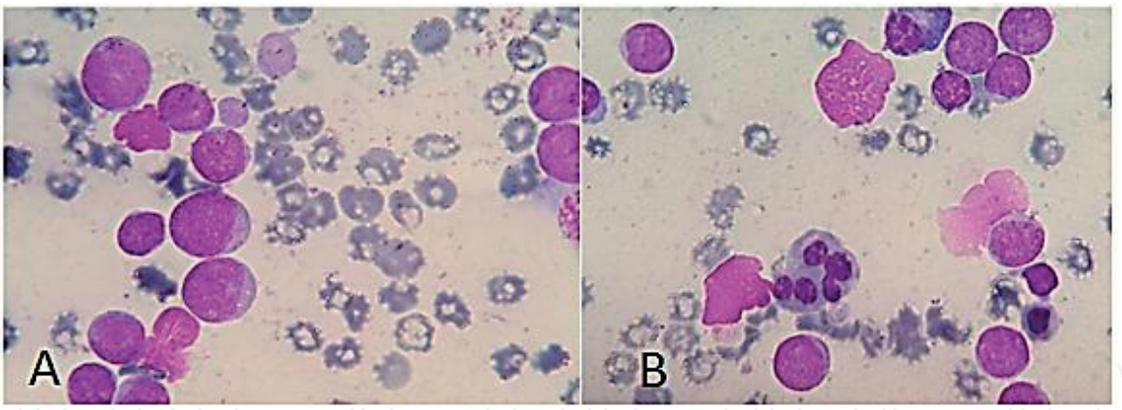

Figure 4.
Bone marrow aspirate from a patient with AML: in picture (A), on the right middle part, a myeloblast displaying a Auer rod surrounded by multiple myeloblasts is shown, and in picture (B), a dysplastic oxyphilic erythroblast showing nuclear abnormalities(multiple nuclei, some being incompletely divided) along with myeloblasts (MGG X 1000) is shown.

Myeloblasts are described as intermediate sized cells, ranging from 14 to 18 μm (when compared to a neutrophil that has the size between 10 and 15 μm) with a nucleo-cytoplasmatic ratio largely in favor of the nucleus (ranging from 4/1 to 5/1 in favor of the nucleus) containing a large, mostly oval-shaped nucleus containing very fine nonagreggated chromatin in which 2 or more nucleoli can usually be seen; the cytoplasm is strongly basophilic and may contain Auer rods [7, 8].

Auer rods are rod-shaped crystalline structures that are derived from primary granules of the myeloid cells. They are mainly reported in AML. They were first reported by John Auer in 1906 and, interestingly, were considered to be inclusions inside lymphoblasts. In the current day, they are considered of diagnostic importance to indicate both the linage and the neoplastic nature of the condition observed [7, 8] (**Figure 4**).

The European Leukemia Network recommends genetic testing of people diagnosed with acute myeloid leukemia, in order to have a complete picture of the risk initiation for each patient, thus being able to use the most appropriate strategy in the fight with this disease. The main genetic markers that can be used are: t (8;21) (q22; q22.1)/RUNX1-RUNX1T1, t (15;17)/PML-RARA, t (9;11) (p21.3; q23.3)/MLLT3-KMT2A, other types of translocation may occur that affect KMT2A genes, t (6;9) (p23; q34.1)/DEK-NUP214, inv (3) (q21.3; q26.2) or t (3;3) (q21.3; q26.2)/GATA2, inv (16) (p13.1q22) and/or t (16;16) (p13.1; q22)/CBFB-MYH11, MECOM, chromosome loss 5/5q, 7, or 17/17p, mutations in CEPBA (biallelic), NPM1, RUNX1, ASXL1 and TP53, and internal tandem duplications (ITD) in the FLT3 gene [9–11].

The most common Next-Gen Sequencing platform currently on the market are offered by Illumina (San Diego, CA, USA), being represented by iSeq100, miniSeq, miSeq, nextSeq System, HiSeq2500, HiSeqX Ten, and NovaSeq, and Thermo Fisher Scientific (Waltham, MA, USA) offers the Ion Proton System, Ion PGM System, Ion S5 System, Ion S5 XL System, Ion GeneStudio S5 System, and the HID GeneStudio S5 System.

For myeloid disease, various NGS Gene panels were designed and validated. Those panels are represented by the proposal of:

a. SureSeq myPanel™ NGS Custom AML (Oxford Gene Technology, Begbroke, Oxfordshire, UK);

b. Leuko-Vantage Myeloid Neoplasm Mutation Panel (Quest Diagnostics, Madison, NJ, USA);

c. AmpliSeq® Myeloid Sequencing Panel (Illumina); and

d. Human Myeloid Neoplasms Panel (Qiagen, Venlo, the Netherlands).

A comparison between these panels is described succinctly by Matynia et al. [12] in **Table 1**.

From this panel, it is recommended to choose the genes to be analyzed according to the choice of the diagnostician. This panel also includes RNA markers for fusion driver genes and expression genes that are not completely listed. The genes listed here come from three other panels and represent combinations between the genes listed. The Web source of this panel presents no other information about the hotspot or the complete gene [13, 14].

The use of NGS techniques can detect mutations in the pretreatment phase, thus having utility in assessing the risk of these patients, establishing the prognosis, making an appropriate and personalized therapeutic decision for each patient, and may even lead to changes in the classification of these types of diseases by WHO.

3. Chronic myeloid leukemia: patho-molecular mechanism and diagnosis

Chronic myeloid leukemia is a hemato-oncologic disease, in which a monoclonal line proliferates. The cells are derived from the hematopoietic stem cell, being characterized by aberrant expression of the BCR/ABL oncogene, arising from the chromosomal translocation t (9;12) (q34; q11). This mutation leads to disruption of the fusion protein, increasing the activity of tyrosine kinase, which will lead to proliferation out of control of the myeloid line [15].

This pathology represents about 15–20% of the cases of leukemia in adults, the main clinical features being represented by leukocytosis, the deviation to the left of the leukocyte formula, with splenomegaly, having an progression in three phases: the initial chronic phase, which can last several years, manifested by increasing the number of myeloid cells, but will retain their differentiation capacity and functions, most patients being asymptomatic. The second phase is an intermediate step of acceleration that can last from several months to several years, difficult to diagnose, being most often discovered following routine blood checks, which highlight the increase in the number of immature and frequent blood cells associated symptoms. In the final blastic phase, immature blood cells predominate, and the hope of survival is several months. In this phase, the genetic instability increases, accumulating these defects, and together with them will increase the resistance to drug therapy (**Figure 5**) [16].

The first line in the diagnosis of CML, right after the cell blood count (CBC) done by an automated analyzer, is the blood smear.

Morphology of the peripheral blood smear plays a crucial role in CML due to the differential diagnosis. A well-done blood smear could exclude a leukemoid reaction (in CML, basophilia is found) and can fastly assess the severity of the disease (high basophil count could be a clue that the disease is heading toward an accelerated phase that, eventually, turns into acute leukemia) as well as a blast crisis (blasts more than 20% that shows the change into acute leukemia) [17].

Qiagen Human Myeloid Neoplasms Panel				
ASXL1 (full)	NPM1 (full)	CBL (full)	SETBP1 (full)	GATA2 (full)
CEBPA (full)	NRAS (full)	CSF3R (full)	SF3B1 (full)	HRAS (full)
DNMT3A (full)	RUNX1 (full)	ETV6 (full)	SRSF2 (full)	IKZF1 (full)
FLT3 (full)	TET2 (full)	EZH2 (full)	ZRSR2 (full)	KMD6A (full)
IDH1 (full)	TP53 (full)	GATA1 (full)	ABL1 (full)	MYC (full)
IDH2 (full)	U2AF1 (full)	JAK2 (full)	BRAF (full)	MYD88 (full)
KIT (full)	WT1 (full)	MPL (full)	CREBBP (full)	NF1 (full)
KMT2A (full)	BCOR (full)	PHF6 (full)	DDX41 (full)	NTRK3 (full)
KRAS (full)	CALR (full)	PTPN11 (full)	EGFR (full)	PDGFRA (full)
PRPF8 (full)	RB1 (full)	SH2B3 (full)	SMC1A (full)	STAG2 (full)
Total	50 genes available			
Illumina AmpliSeq Myeloid Panel				
ASXL1 (full)	NRAS (hotspot)	—	ZRSR2 (full)	MYC (expression)
CEBPA (full)	RUNX1 (full)	EZH2 (full)	ABL1 (hotspot)	MYD88 (hotspot)
DNMT3A (hotspot)	TET2 (full)	—	BRAF (hotspot)	NF1 (full)
FLT3 (hotspot)	TP53 (full)	JAK2 (hotspot)	CREBBP (fusion)	NTRK3 (fusion)
IDH1 (hotspot)	U2AF1 (hotspot)	MPL (hotspot)	—	PDGFRA (fusion)
IDH2 (hotspot)	WT1 (hotspot)	PHF6 (full)	EGFR (fusion)	PRPF8 (full)
KIT (hotspot)	BCOR (full)	PTPN11 (hotspot)	GATA2 (hotspot)	RB1 (full)
KMT2A (fusion)	CALR (full)	SETBP1 (hotspot)	HRAS (hotspot)	SH2B3 (full)
KRAS (hotspot)	CBL (hotspot)	SF3B1 (hotspot)	IKZF1(full)	SMC1A (expression)
NPM1 (hotspot)	CSF3R (hotspot)	SRSF2 (hotspot)	—	STAG2 (full)
Total	46 genes available			
Quest Diagnostics LeukoVantage Panel				
ASXL1	NRAS	—	ZRSR2	—
CEBPA	RUNX1	EZH2	—	—
DNMT3A	TET2	GATA1	—	—
FLT3	TP53	JAK2	—	—
IDH1	U2AF1	MPL	DDX41	—
IDH2	WT1	—	—	—
KIT	—	PTPN11	—	—
KMT2A	CALR	SETBP1	—	—
KRAS	CBL	SF3B1	—	—
NPM1	CSF3R	SRSF2	KMD6A	—
Total	30 genes available			

Oxford Gene Technology SureSeq myPanel NGS Custom AML				
ASXL1 (full)	NRAS (full)	ETV6 (full)	—	—
CEBPA (full)	RUNX1 (full)	—	—	—
DNMT3A (full)	TET2 (full)	GATA1 (full)	—	—
FLT3 (full)	TP53 (full)	—	—	—
IDH1 (full)	U2AF1 (full)	—	—	—
IDH2 (full)	WT1 (full)	PHF6 (full)	—	—
KIT (full)	BCOR (full)	—	—	—
KMT2A (full)	—	—	—	—
KRAS (full)	—	—	—	—
NPM1 (full)	—	—	—	—
Total	20 genes available			

The term "full" indicates all exons, and the term "hotspot" indicates hotspot exons (unmentioned here).
The term "fusion" indicates the RNA fusion partner; this genes has not been analyzed as DNA sequence, and the panel does not include all the RNA fusion partners.
The term "expression" indicates the analyses of quantification of gene expression at mRNA level, those genes not being analyzed at the DNA level.

Table 1.
Overview of commercially available NGS panels for AML with a list of included genes [12].

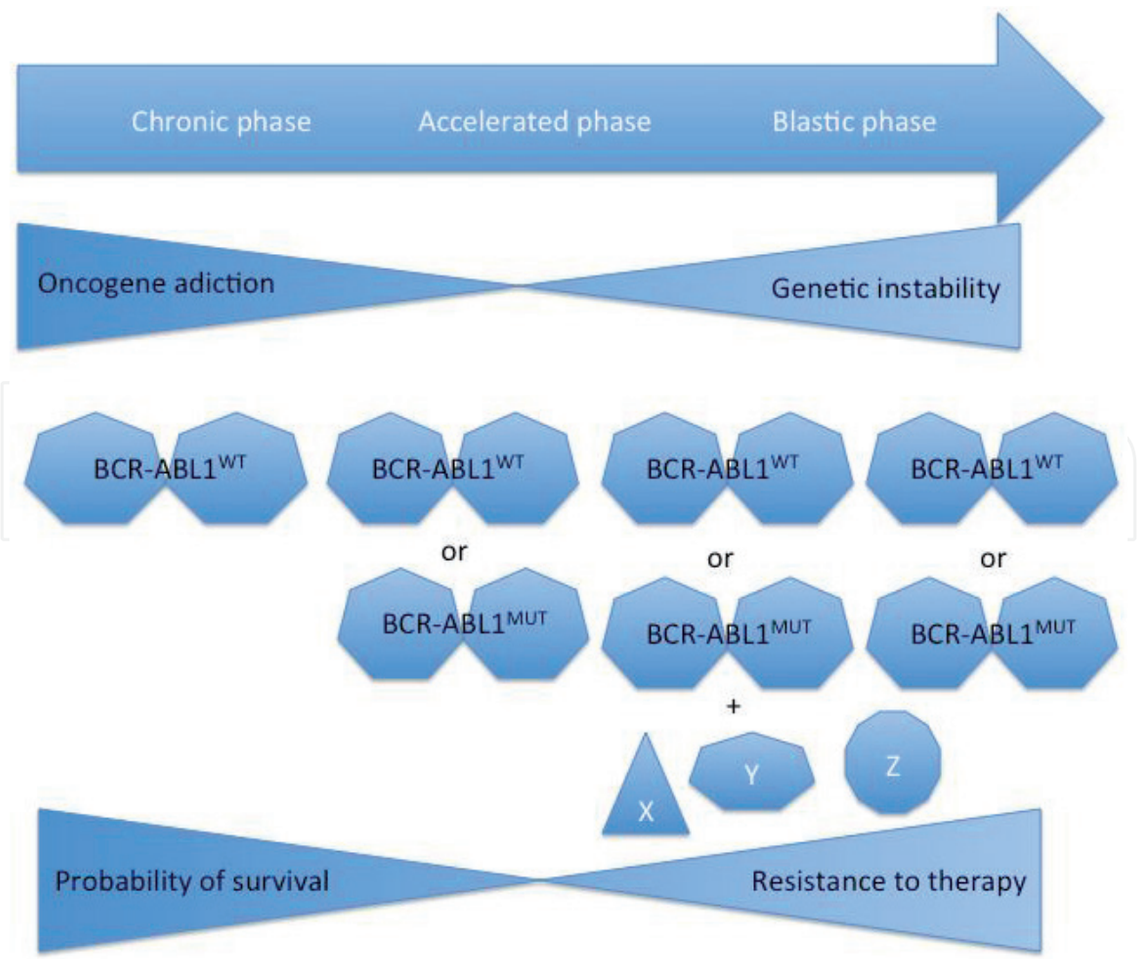

Figure 5.
Evolution of chronic myeloid leukemia [16].

The bone marrow aspirates in CML are hypercellular with an expansion of the granulocytes (e.g., neutrophils, eosinophils, and basophils) and their progenitor cells. In most cases, megakaryocytes are prominent and most often their size is increased (**Figure 6**) [18].

Most cases of chronic myeloid leukemia show the Philadelphia chromosome, which appeared after alteration of chromosome 22, produced by a reciprocal translocation t (9;22) q (34; 11), thus forming the BCR-ABL1 fusion gene, which became the main one diagnostic marker in chronic myeloid leukemia [19].

The phenotype types associated with this condition are closely correlated with the size of the proteins encoded by the different transcripts of the BCR-ABL1 fusion gene, thus noting that the most frequent rearrangements are represented by b2a2, followed by b3a2, following the rare alterations considered to occur in less than 2% of all cases of chronic myeloid leukemia. Depending on the rearrangements that have been undergone, theories have been issued that claim that transcript b2a2 is responsible for lowering the optimal response rate, and transcript b3a2 is associated with better therapeutic response and longer post treatment remission [20].

NGS is now commonly used to detect mutations in the ABL1 kinase. This represents a mechanism of CML resistance to TKIs, being about half percent of the acquired resistance for CML cases where treatment failed. It has been shown that many unique and different kinase domain mutations are associated not only with imatinib resistance but also with resistance to nilotinib (Y253H, E255K, E255V, F359V, and F359C), dasatinib (V299L, T315A, F317L, F317I, F317V, F317V, F317V, and F317C), or bosutib (Y253H, V299L, and F317V) [21].

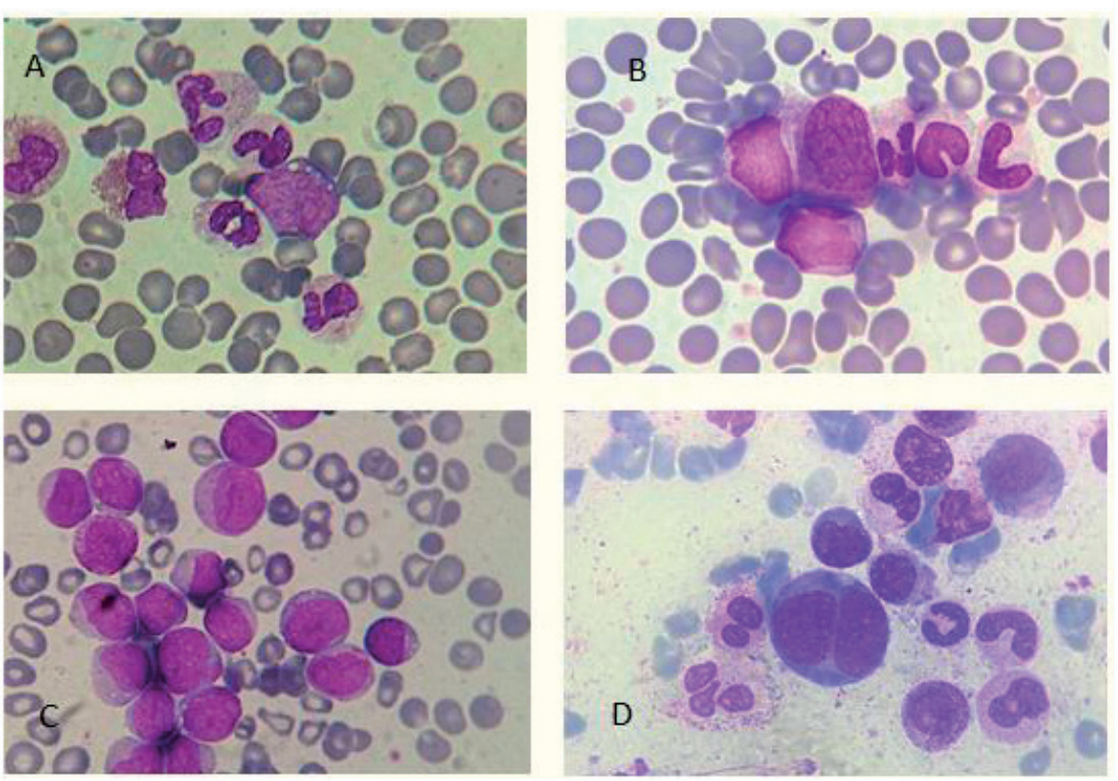

Figure 6.
Chronic myeloid leukemia staining MGG, magnification stage 1000X: (A) Patient with chronic myeloid leukemia, a blast accompanied by dysplastic granulocyte precursors (above the blast, there is unsegmented hypogranular neutrophil, and above, we see an abnormal segmented neutrophil). (B) Patient with chronic myeloid leukemia that displays most of granulocyte precursors (band cell, metamyelocyte, promyelocyte, a myelocyte with nucleo-cytoplasmatic asynchronism and below a blast cell). (C) Blast crisis in chronic myeloid leukemia. (D) Bone marrow aspirate of a patient with CML showing erythroid dysplasia (basophilic giant binucleated erythroblast) accompanied by several hypogranular granulocyte precursors.

Even if these specific TKI mutations are present in less than 10% of the cases in which the treatment fails, the identification followed by the characterization of these mutations is especially important in choosing the optimal type of TKI that could be used even when the resistance was acquired.

In the hybridization-based NGS technique, artificial oligonucleotides specially designed for BCR and ABL1 marker sequences are used. This specific amplification is followed by sequencing. By in silico analyzes, with the help of software, the fusion junctions are identified on these sequences, whether they are determined by different types of chromosomal structural rearrangements such as chromosomal translocations, inversions, or deletions.

4. Conclusions

In the last decades, the correct evaluation of MRD has been of major importance for the superior management of the treatment with TKIs for patients suffering from CML. This process became easier and more accurate than in the case of other hematological malignancies, precisely because the fundamental pathogenetic mechanism of this disease was studied and deciphered, which led to the use of the BCR-ABL1 transcript as the main target of all MRD tests.

An important point in the identification and treatment of CML was the development and adoption of NGS instruments in the clinical field to evaluate mutations undergone in the ABL1 kinase domain, as these mutations are responsible for resistance to the treatment of TKIs in any phase of the disease, either it is chronic or at an advanced stage. Although the Sanger sequencing method has been the most commonly used, the development of the NGS technique, which has a much higher sensitivity, enabling the detection of mutations at the subclonal level, and compound mutations that are responsible for resistance to ponatinib, has led to notable advances in diagnosis and treatment of this disease.

In the near future, it is expected that the use of SNG will be increasingly adopted for patients whose first line of treatment fails but also for those who do not respond optimally to the additional line of treatment. However, there is still much to be done in this area; for example, for allogeneic transplantation, there is no NGS-generated data available, here Sanger is still the technique used.

The existing scientific data so far indicate that the successful therapeutic management of patients with CML is, without doubt, the close collaboration between biologists, technicians, and doctors, which involves primarily the use of scientific evidence data and innovative techniques such as those based on DNA analysis. Existing and ongoing networks, online databases, and ongoing development of methods and equipment will help to achieve these goals.

Appendices and nomenclature

FDA	Food and Drug Administration
NGS	next-generation sequencing
IVDs	in vitro-based diagnostics
AML	acute myeloid leukemia
MGG	May-Grunwald-Giemsa stain
RBC	red blood cells
WHO	World Health Organization
CML	chronic myeloid leukemia
TKIs	tyrosine kinase inhibitors

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Author details

Oana Maria Boldura[1], Cristina Petrine[2], Alin Mihu[2] and Cornel Balta[2,3]*

1 Faculty of Veterinary Medicine, Department of Chemistry, Biochemistry and Molecular Biology, Banat University of Agricultural Sciences and Veterinary Medicine "King Mihai I of Romania", Timisoara, Romania

2 Emergency County Clinical Hospital, Arad, Romania

3 Institute of Life Sciences, Vasile Goldis Western University of Arad, Romania

*Address all correspondence to: baltacornel@gmail.com

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