Class III Receptor Tyrosine Kinases in Acute Leukemia – Biological Functions and Modern Laboratory Analysis

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Supplementary Issue: Tyrosine Kinases in Cancer

ABSTRACT: Acute myeloid leukemia (AML) is a complex disease caused by deregulation of multiple signaling pathways. Mutations in class III receptor tyrosine kinases (RTKs) have been implicated in alteration of cell signals concerning the growth and differentiation of leukemic cells. Point mutations, insertions, or deletions of RTKs as well as chromosomal translocations induce constitutive activation of the receptor, leading to uncontrolled proliferation of undifferentiated myeloid blasts. Aberrations can occur in all domains of RTKs causing either the ligand-independent activation or mimicking the activated conformation. The World Health Organization recommended including RTK mutations in the AML classification since their detection in routine laboratory diagnostics is a major factor for prognostic stratification of patients. Polymerase chain reaction (PCR)–based methods are well-validated for the detection of fms-related tyrosine kinase 3 (*FLT3*) mutations and can easily be applied for other RTKs. However, when methodological limitations are reached, accessory techniques can be applied. For a higher resolution and more quantitative approach compared to agarose gel electrophoresis, PCR fragments can be separated by capillary electrophoresis. Furthermore, high-resolution melting and denaturing high-pressure liquid chromatography are reliable presequencing screening methods that reduce the sample amount for Sanger sequencing. Because traditional DNA sequencing is time-consuming, next-generation sequencing (NGS) is an innovative modern possibility to analyze a high amount of samples simultaneously in a short period of time. At present, standardized procedures for NGS are not established, but when this barrier is resolved, it will provide a new platform for rapid and reliable laboratory diagnostic of RTK mutations in patients with AML. In this article, the biological and physiological role of RTK mutations in AML as well as possible laboratory methods for their detection will be reviewed.

KEYWORDS: AML, Tyrosine kinase, mutations, diagnosis, laboratory analysis

Introduction

Receptor tyrosine kinases (RTKs) are involved in different steps of neoplastic development and progression. Their signaling influences the growth, differentiation, adhesion, motility, and death of cells.[1] RTKs are divided in 20 subfamilies including class III RTKs. Mutations in class III RTKs have a major impact on leukemic transformation of acute myeloid leukemia (AML) cells. Class III RTKs include among others *c-Kit*, *CSF1R* (colony-stimulating factor 1 receptor), *FLT3* (fms-related tyrosine kinase 3), and *PDGFR α/β* (platelet-derived growth factor receptor α/β).[2] These molecular receptors are membrane-bound enzymes consisting of an extracellular ligand-binding domain (ED), a juxtamembrane domain (JMD), a highly conserved intracellular tyrosine kinase domain (TKD), and a C-terminal tail (Fig. 1).[3] In the nonactivated state, class III RTKs reside in the cell membrane as auto-inhibited monomers and in some cases as preformed dimers. They are activated by dimeric short-chain α-helix bundle cytokines and upon activation induce essential signals for the development and homeostasis of hematopoietic cells.[4–6] *PDGFR α/β* is activated by a different ligand, which contains an all–β-strand cysteine-knot fold. *PDGFR α/β* regulates diverse functions involved in mesenchymal processes as bone formation, tissue repair, and fibroblast proliferation.[7,8]

Under physiological conditions, RTKs prevent deregulated proliferation and influence the sensitivity of cells toward apoptotic signals. In cancer cells, the genetic and epigenetic modification of RTKs induces a selection advantage of altered cells leading to rapid and uncontrolled proliferation. Genetic mutations of class III RTKs have a major impact on the prognosis of AML patients. They are included in the World Health Organization classification of AML as a significant prognostic factor. Since they influence the treatment decision, their detection in routine laboratory is important in patients with AML at diagnosis and in follow-up samples.[9–11]
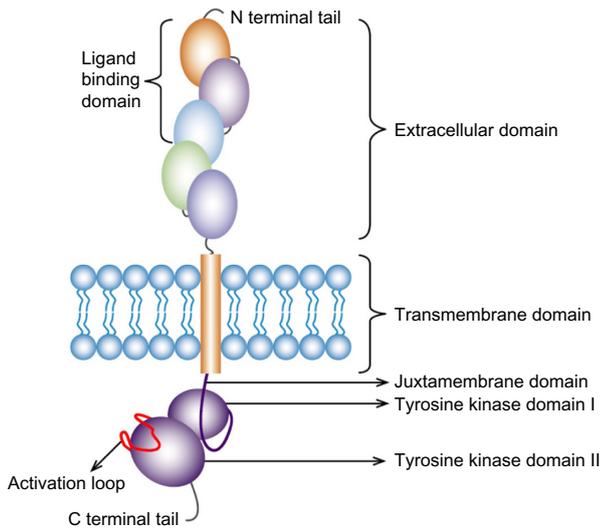
Figure 1. Molecular composition of class III RTKs. Class III RTKs consist of an ED containing five IgG-like domains. The outer three are responsible for ligand binding. A linker connects the ED with the transmembrane domain (TMD), which is localized in the plasma membrane of the cell. The JMD lies directly behind the TMD (purple line). The conserved TKDI and TKDII follow the JMD and are separated by a kinase insert. The TKDII contains the activation loop (red line), which enables binding of downstream substrates when an activated conformation is established. Furthermore, RTKs are flanked by an N-terminal and C-terminal tail. Adapted by permission from Macmillan Publishers Ltd, Nat. Rev Cancer. Verstraete K and Savvides SN. Extracellular assembly and activation principles of oncogenic class III receptor tyrosine kinases. *Nat Rev Cancer.* 2012;12(11):753–766. Copyright 2012.[2]

Biochemical Activation Mechanism

Activation and downstream signaling of RTKs essentially depend on binding of appropriate ligands to the ED. To ensure that signals by RTKs are only transmitted when their action is needed for the cell, their activity is inhibited by auto-inhibition when no ligand is bound. The auto-inhibition mechanism differs among RTK families. In class III RTKs, the JMD plays a crucial role for this process. In the inactivated state, the JMD occupies the active site cleft between the N-kinase and C-kinase lobes generating an auto-inhibitory structure. This prevents the activation loop from moving to its active conformation. Ligand-dependent activation is contingent on the resolution of the auto-inhibitory conformation by phosphorylation of conserved tyrosine residues of the JMD. The auto-inhibition mechanism acts in *cis*, whereas activation switches the conformation to *trans* (Fig. 2).[12,13]

Ligand binding takes place in the ED. Ligands are extracellular signal molecules that induce receptor dimerization and subsequent activation and phosphorylation of different tyrosine residues. Either one or two ligands bind to two receptor molecules. Oligomerization of the receptor increases its local concentration, supporting the efficient transphosphorylation of tyrosine residues in the catalytic domain of the activation loop. Stimulation by ligands induces the transphosphorylation of tyrosine residues in the JMD, leading to the release of inhibitory intramolecular interactions and swing out of the tyrosine side chains. Establishment
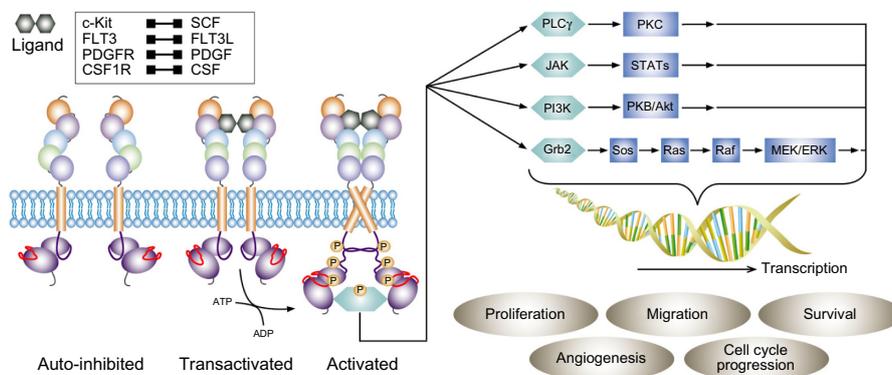

Figure 2. Activation and downstream signaling of class III RTKs. In the inactivated state, RTKs are auto-inhibited by the JMD (purple line) in *cis*. Ligand binding occurs in the ED indicated by orange, purple, green, and blue circles. Ligands of RTKs discussed in this review are specified in the upper left box and the ligand is depicted as a grey double-hexagon. Ligand binding induces the oligomerization of the receptor. Thereby, the IgG-like domains of the ED rotate to form an efficient receptor–ligand complex. This induces transphosphorylation of tyrosine residues in the TKD (purple circles) and JMD (purple line). Phosphorylation of the JMD leads to the release of the inhibitory conformation and to the swing out of the tyrosine side chains. The phosphate is generated through transformation of ATP to adenosine diphosphate. Thereby, the conformational change (into *trans*) of the RTK processes from a transactivated to a fully activated state. In the activated state, the titling angle of the ligand shifts when the ternary complex is generated with the exception of PDGF. In addition, the activation loop (red line) establishes an active conformation enabling the binding of substrates (turquoise hexagon) and ATP. Downstream signaling is achieved by phosphorylation (indicated as orange circles with P) of signaling molecules described in the right part of the figure. This includes PLCγ, JAK/STATs, PI3K, and the MAPK signaling pathway. These signaling pathways regulate the transcription of various genes implicated in cell proliferation, migration, survival, angiogenesis, and cell cycle progress. Constitutive activation of RTKs by different mutations would lead to constitutive phosphorylation of downstream effectors and deregulation of the transcription of genes implicated in cell proliferation and others. Adapted by permission from Macmillan Publishers Ltd, Nat. Rev Cancer. Verstraete K and Savvides SN. Extracellular assembly and activation principles of oncogenic class III receptor tyrosine kinases. *Nat Rev Cancer.* 2012;12(11):753–766. Copyright 2012.[2]

Abbreviatons: Grb2, growth factor receptor–bound protein 2; Sos, son of sevenless; Raf, rat fibrosarcoma; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase.

of an active conformation of the activation loop enables the binding of substrates and adenosine triphosphate (ATP). Thereby, magnesium-ATP is transferred to tyrosine residues on the receptor itself and on downstream cellular signaling proteins.[14–16]

The catalytic domain is the strongest conserved domain of RTKs. It contains different binding domains for signaling proteins with *SH2* (src-homology 2) and *PTB* (phosphoty-rosine-binding) domains, which recruit further effector molecules containing *SH2*, *SH3* (src-homology 3), *PTB*, or *PH* (pleckstrin homology) domains. This provides the basis for the assembly of a signaling complex to the activated RTK, inducing a cascade of biochemical signals that activate or repress genes. Major molecules that are influenced by this mechanism are *Ras/MAPK* (rat sarcoma/mitogen-activated protein kinases), *PI3K* (phosphoinositide 3-kinase), *PLC-γ* (phospholipase C-γ), *JAK* (janus kinase), *STATs* (signal transducers and activators of transcription) and *NF-κB* (nuclear factor kappa-light-chain enhancer of activated B-cells). Thereby *Ras/MAPK*, *STAT*, and *PI3K* are the major oncogenic signals in AML.[3,17,18]

During the normal signaling process, RTKs migrate to the plasma membrane. This leads to internalization of RTKs by a clathrin-coated invagination causing degradation by lysosomal enzymes or recycling. The internalization induces dissociation of the ligand–receptor complex, which terminates the RTK signaling.[19]

Oncogenic Activation Mechanisms

Oncogenic activation of RTKs is induced either by mutations in the ED and activation loop or by abnormal chromosome translocations resulting in gain-of-function. Chromosomal breakpoints targeting RTKs generate fusion proteins. The partner protein is joined to the entire catalytic domain leading to constitutive activation of the RTK moiety. Activating point mutations can occur in different domains, whereby the major portion of these mutations belongs to class I mutations according to the two-hit model for AML development (Fig. 3).[20,21]

Mutations in the ED. The ED contains different structures including immunoglobulin-like (IgG-like) domains, cysteine-rich domains, fibronectin type III–like domains, and
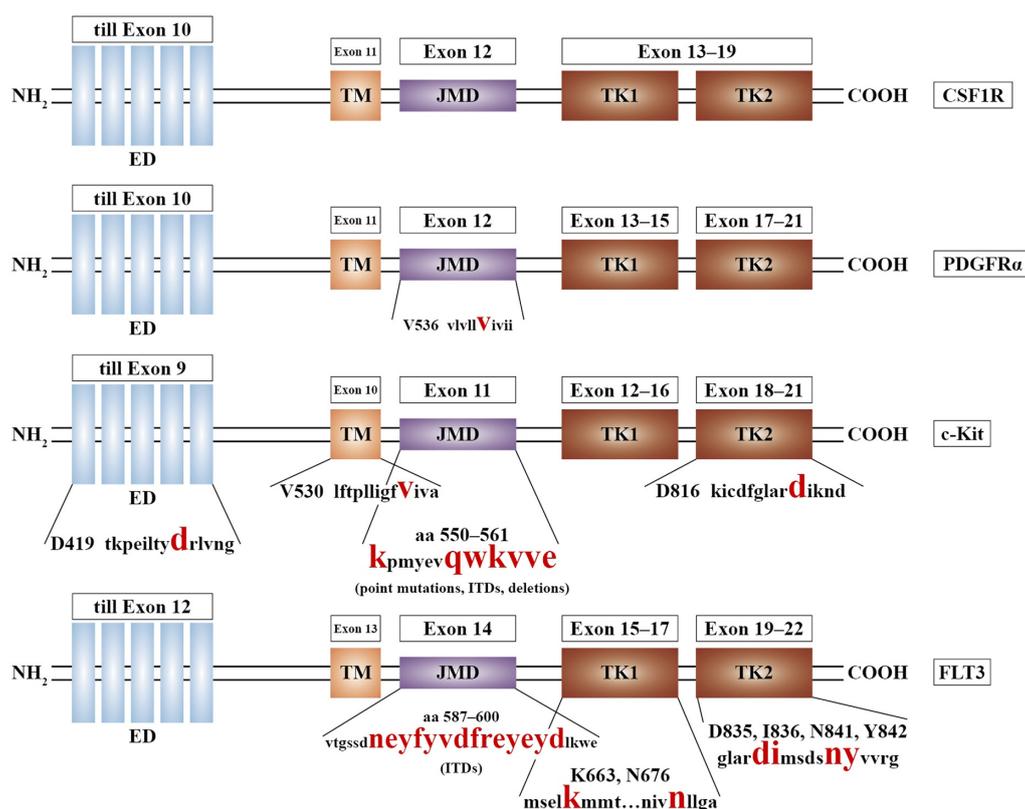
Figure 3. Genomic organization and mutational spots of class III RTKs. Exon localization of the protein domains of *CSF1R*, *PDGFRα*, *c-Kit*, and *FLT3* is shown. In addition, amino acid sequences of mutational spots are described (indicated by lines at the corresponding domain). The affected amino acids are marked red. In *PDGFRα*, a mutation at aa V536 was detected in the JMD. In *c-Kit* mutations in the ED, TM domain, JMD, and TK 2 domain were detected. These are mainly point mutations (D419, V530, and D816), with the exception of the JMD. In this domain also, insertions/deletions and ITDs were detected (aa 550–561). In *FLT3* mutations were detected in the JMD and TK domains. Mutations of the JMD are mostly ITDs between aa 587–600. In the TK domains, only point mutations were reported (K663, N676, D835, I836, N841, Y842). D835 is present in around 10% of patients with AML, whereas the other point mutations are rather rare.
Abbreviations: TM, transmembrane; TK1/2, tyrosine kinase 1/2.

others. Because of these manifold structural elements, the ED is able to recognize a wide variety of extracellular stimuli with sufficient selectivity and affinity.[22,23]

Mutations in the ED induce constitutive activation of the RTK without the presence of extracellular ligands. Thereby, the IgG-like domain has a major impact on the stability of ligand-induced dimerization. Mutations in this domain induce a conformational change, which enables dimerization and activation.[24]

In AML, *c-Kit* mutations in exon 8 were identified. The in-frame deletion and insertion induced a constitutive loss of D419, eventhough the detailed mechanism of activation remains unclear.[25] In addition, mutations in the ED can lead to a shift in the substrate specificity and for example enhance the affinity toward ATP. It was shown that wild-type *c-Kit* and mutated *c-Kit* phosphorylate the *EGFR* (epidermal growth factor receptor) peptide, but mutated *c-Kit* phosphorylates an *ABL* peptide with a much higher efficiency as compared to the wild-type form.[26] Furthermore, chromosomal translocations can lead to artificial dimerization by fusion with a dimerization domain. For example, a reciprocal translocation with *FGFR1* (fibroblast growth factor receptor 1) was identified for *PDGFRβ*. This fusion imitates the ligand-induced dimerization by additional dimerization motifs like the *ETV6* helix-loop-helix domain, *BCR* coiled-coil domain, and a proline-rich domain in *ZNF198*.[27]

Mutations in the JMD. The JMD of RTKs varies in length and is not essentially required for kinase activity. In addition, there is a lack of sequence similarity for this domain. As previously described, the JMD is a negative regulator domain due to its importance for the generation of an auto-inhibitory conformation. Furthermore, the C-terminal tail provides additional docking sites for signaling proteins. Class III RTKs share approximately 30 residues in the JMD, but their structure can vary.[17] For example, *FLT3* is separated into three domains. The juxtamembrane (JM)-binding motif consists of seven amino acids (aa) and is almost completely buried into the cleft between the N- and C-kinase lobes. It interacts with different components of the inactivation and activation machinery. The JM switch motif is assembled by an antiparallel β-sheet and is associated with the C-kinase lobe. The JM zipper/linker protein is located at the C-terminus of the JMD and associated with the N-kinase lobe.[28]

Point mutations, deletions, or insertions of the JMD are found in *c-Kit*, *FLT3*, and *PDGFRα/β*. Mutations disrupt the ability of the JMD to form an α-helix followed by a three-stranded β-sheet leading to the loss of the auto-inhibitory effect.[29] *c-Kit* mutations were identified in position D550–580, leading to disruption of the regulatory conformation and constitutive activation of the RTK. Substitutions and deletions of *c-Kit* reside in most instances in the N-terminal to the JM-binding region.[26,30] Leukemic cells with mutant *c-Kit* can be targeted by different *c-Kit* inhibitors. However, it was also reported that interaction with bone marrow stromal cells

protects leukemic cells from the inhibitory effects of anti–*c-Kit* drugs. One of the main factors responsible for this rescue seems to be granulocyte–colony-stimulating factor.[31]

FLT3 mutations in the JMD are mainly in-frame internal tandem duplications (ITDs) that duplicate a fragment of the coding sequence. The length and position of the ITD can differ between AML patients ranging from 3 bp to more than 400 bp (4–68 aa). Mostly, the tyrosine-rich region 587-N-E-Y-F-Y-V-D-F-R-E-Y-E-Y-D-600 in exon 14 is involved. This affects the conformation of the JM binding and switching motif, inducing the loss of the inhibitory effect.[14,20,32] Despite the development of therapeutic agents against *FLT3* mutations, not all patients benefit from this target therapy because of limited efficiency and drug resistance. Chatterjee et al identified constitutive phosphorylation of STAT5 downstream of *FLT3*-ITD and also *c-Kit* D816V mutations. Furthermore, they reported that focal adhesion kinase, Ras-related C3 botulinum toxin substrate 1 (*Rac1*), and the serine/threonine-protein kinase *PAK 1* regulate the nuclear translocation of active STAT5 in *FLT3*- and *c-Kit*–oncogenic cells and could therefore depict potential therapeutic targets,[33] eventhough other molecules such as Bruton tyrosine kinase were also identified as important downstream effectors in FLT3-ITD mutant cells, promoting cell survival and proliferation.[34]

In *PDGFR*, a point mutation at the position V536A was identified.[35]

Mutations in the TKD. The TKD catalyzes transfer of γ-phosphate from ATP to the hydroxyl group of different target proteins. In contrast to the JMD, the catalytic core of the TKD is highly conserved between different RTKs. The TKD displays a special structure consistent of the activation loop, a peptide segment of 20–30 aa, which is flanked by a small N-terminal and a large C-terminal lobe. This composition provides the flexibility and mobility of the activation loop, which is needed for binding of substrates to this region. In some RTKs, like *PDGFRα/β*, the TKD contains a large insert that provides docking sites for downstream signaling proteins. Unlike the JMD, the function of the TKD is essential for RTK signaling. However, when no activation signal is present, it also participates in the auto-inhibitory regulation by folding into the cleft between the N-kinase and C-kinase lobes. This prevents substrate binding to the active site. The exact mechanism of auto-inhibition of TKD can vary in different RTKs.[3,28,36]

The phosphorylation of an activation loop tyrosine is the key effect that induces conformational change of the TKD and enables substrate binding. Furthermore, the phosphorylation state of one to three tyrosines in the central region of the TKD correlates directly with the catalytic activity of the RTK.[22]

Most mutations occur in asparagine residues of the TKD, leading to the independent dimerization of this domain, regardless of substrate binding in the ED. Mutations seem to mimic the conformational change, which is induced

by tyrosine phosphorylation and therefore cause constitutive activation of the RTK.[37]

A mutation in D816 was identified in *c–Kit*. The activation motif L-A-R-D-I adjacent to the D-F-G motif utilizes a helix structure, which is rotated around the asparagine residue of the D-F-G motif upon activation of *c–Kit*. This transforms the activation motif into a β-strand, creating a structured organization for protein ligation. It is assumed that D816 mimics this structural change and induces constitutive activation. The aspartic acid can be mutated to tyrosine, histidine, asparagine, or valine with similar consequences.[38–40] Agarwal et al reported that oncogenic activity of D816 mutants relies on the efficiency of the activation loop tyrosine Y823. They found an impaired transforming capacity of leukemic cells with mutations in both D816 and Y823. Furthermore, deletion of Y823 function led to an increased sensitivity of leukemic cells toward apoptosis and prohibited the phosphorylation of the downstream signaling molecule STAT5.[41]

The second most common mutation in *FLT3* is a point mutation at D835 in the activation loop. The aspartic acid is changed either to tyrosine or histidine. Further mutations in *FLT3* in this region were also described (Y842C and N841I). In contrast to *FLT3*-ITD, prognostic influence of point mutations is still not well established and remains controversial.[42–44] Generally, *FLT3* mutations are a poor prognostic marker. One study identified better prognosis of AML patients with *FLT3*-D835 mutation as compared to AML patients with *FLT3*-ITD.[45] However, both mutations induce the cytokine-independent growth of hematopoietic cells caused by the aberrant phosphorylation of downstream signaling proteins. It is also assumed that differences in the signal transduction between *FLT3*-ITD and D835 could be responsible for the diverse prognostic influence of these mutations.[23,46]

Detection of RTK Mutations

Mutations in RTKs play a major role for the course of AML disease. Therefore, their detection for diagnosis of AML as well as their analysis at relapse and in follow-up samples has a great impact on treatment decisions and response to therapy. *FLT3* and *c–Kit* are particularly important in AML.

Gain-of-function mutations of *c–Kit* are strongly associated with the core-binding factor (CBF) AML.[47] Various studies analyzed the prognostic impact of *c–Kit* mutation and suggest that its clinical significance could be dependent on mutation type, patient characteristics, and existing chromosomal translocations.[48–50] Furthermore, an adverse prognostic influence of *c–Kit* mutation was reported in AML patients harboring inv(16) or t(8,21).[51] Mutation frequencies vary between different cytogenetic groups. In patients with inv(16), activating *c–Kit* mutations are present in 30%–40% of cases. In contrast, incidence of *c–Kit* in patients with t(8,21) is more variable and ranges from 20% to 30%.[52] In addition, the National Comprehensive Cancer Network guidelines have classified AML patients with CBF aberrations and *c–Kit* mutations as an intermediate-risk group. Interestingly, higher expression levels of *c–Kit* were also detected in patients with AML.[53] Ahmadi et al suggested that screening of *c–Kit* expression using FACS should be included in the routine primary panel for the diagnosis of acute leukemia.[54]

FLT3-ITD mutations were reported to have adverse impact on overall survival (OS) due to the high relapse rates.[32] Thereby, *FLT3* mutation status can differ at diagnosis and relapse in patients with AML. Different studies described that gain of *FLT3*-ITD mutations could indicate evolution of a new clone with selective advantages.[55–58] Because of the specific character of neoplastic transformation by *FLT3* mutations, it represents a promising therapeutic target for AML.[59,60] *FLT3*-D835 mutations are present in around 10% of AML cases, whereas *FLT3*-ITD mutations are described in 25% of adult and 15% of pediatric AML cases.[61,62] Therefore, prognostic impact of D835 is more infrequent compared to *FLT3*-ITD mutations. *FLT3* mutations are mostly associated with mutations in other genes such as *DNMT3A* (13.3%) and nucleophosmin 1 (*NPM1*; 6.8%). Different studies reported a negative prognostic impact of *FLT3* mutations compared to patients with discrete mutation of *NPM1*, *DNMT3A*, or other genes.[63–65] Thereby, the prognostic effect of *FLT3* depends on the ratio of mutated to wild-type allele, resulting in a worsened outcome when a high load of mutated allele is present.[66,67] However, this influence also depends on the insertion site. Schlenk et al found that patients harboring a *FLT3*-ITD mutation in the JMD and a high mutation load benefit from allogeneic hematopoietic stem cell transplantation, whereas OS and relapsed free survival could not be improved in patients with insertions in the TKD of *FLT3*.[67]

Activating mutations in *CSF1R* that contribute to pathogenesis of AML have not been described.[68,69] However, activating mutations of downstream components of the *CSF1R* signaling pathway were reported in different hematologic diseases.[68] Mutations in the *NRAS* gene were shown in 40% of patients with AML.[70]

Mutations in *PDGFRα/β* are also rare in AML. Single-nucleotide polymorphisms (SNPs) were identified in 2% of patients with AML.[71,72] It is not clear whether the identified mutations are drivers for AML pathogenesis.[73]

Unlike chronic myeloid leukemia, there are no standard procedures for molecular characterization of patients with AML.[74] Different methods exist for molecular genetic analyses of point mutations and length fragment insertions in DNA, including DNA sequencing, polymerase chain reaction (PCR)–based methods, and capillary electrophoresis. These methods can be applied differentially depending on the detection aim (Table 1).

PCR-based methods. A standard PCR starts with an initial denaturation step followed by a series of 20–40 cycles. The cycles mostly include three different temperature steps

Table 1. Different methods for RTK mutation detection.

METHOD		ADVANTAGES	DISADVANTAGES	USE IN ROUTINE DIAGNOSTIC
PCR-based methods	*FLT3*-ITD (PCR)	Fast Cheap Reliable	No detection of point mutations No quantitative prediction	Yes
	FLT3-D835 (PCR with subsequent endonuclease restriction)	Cheap Reliable Detection of point mutation	Applicability depends on sequence composition Multiple analysis steps No quantitative prediction	Yes
	ARMS-PCR	Fast Cheap Reliable	Applicability depends on sequence composition No quantitative prediction	No
Melting analysis	HRM analysis	High sample throughput Rapid Convenient Semi-quantitative	Sometimes difficult data interpretation Only small amplicon sizes Influence of melting profile by PCR components	No
	in combination with PNAs or LNAs	See HRM analysis Increased selectivity for mutated allele	See HRM analysis	No
	Probe-based melting analysis	See HRM analysis Increased selectivity for mutated allele Reduction of unspecific interactions during PCR	Sometimes difficult data interpretation Only small amplicon sizes	No
DHPLC analysis		High sensitivity Less subjective and labor-intensive Semi-quantitative	Assay reliability strongly depends on analysis temperature validation Sometimes difficult data interpretation	No
Real-time PCR	TaqMan PCR	High sensitivity Quantitative Fast Cheap Reliable	No detection of point mutations Sometimes need for patient specific primers causing increased costs Only small amplicon sizes	Yes
	ASB-PCR	see TaqMan PCR Detection of point mutations Higher selectivity for mutated allele	Time-consuming validation Wild-type control needed Only small amplicon sizes	No
Gene scan		High resolution Semi-quantitative Analysis of larger amplicons possible Cheap Reliable Generation of mutational ratio possible	No detection of point mutations Multiple analysis steps	Yes

DNA sequencing				
Sanger sequencing	Well validated and standardized High accuracy Large amplicon sizes Partly independent of sequence composition	Limited sensitivity Time-consuming Labor-intensive Expensive		Yes
Next generation sequencing	Broad range of possible applications Analysis of high number of mutational spots at the same time High sample throughput More sensitive compared to Sanger sequencing When well validated: High accuracy and reliability	Expensive Reliability of results depends on "read-depth" Reference sequence is needed Difficult data analysis, high bioinformatics know-how needed Difficulties in analysis of repetitive sequences and insertions/deletions		Depends on clinical situation

(denaturation, annealing, and elongation). In addition, a terminal elongation step should be included to ensure that elongation can be performed in full length. The generated PCR products can be identified using agarose gel electrophoresis.[75,76]

In AML diagnosis, this method is mainly used for the identification of *FLT3* mutations. When an ITD insert in *FLT3* is present, the generated PCR fragment is larger as compared to the wild-type allele. These different fragment sizes can be identified by agarose gel electrophoresis. Depending on the primers used, the wild-type allele is seen at approximately 330 bp, whereas the mutated allele is located >330 bp. Because of the heterogeneity of the insert, different patients can display different fragment sizes.

FLT3-D835 can be detected by endonuclease restriction using EcoRV. In the mutated state, a restriction site of the enzyme is lost, leading to the generation of a larger fragment, which can be discriminated by gel electrophoresis. The fragment sizes that can be seen in the agarose gel depend on the PCR primers used for amplification of mutated DNA.[77–79]

In addition, different formats can be applied to identify point mutations in RTKs, for example, ARMS (amplification-refractory mutation system) PCR. This method allows differentiation between wild-type and mutated allele by specific differences in amplification properties of the reactions. The PCR contains four primers, of which two serve as outer control primers and two are located at the mutational spot. One primer anneals to the mutated allele and one to the wild-type allele, producing fragments of different sizes in cooperation with one of the both control primers, respectively. Visualization can also be performed by agarose gel electrophoresis. The wild-type sample shows the wild-type and control band, and the mutated sample shows either the mutant and control band or all three bands (wild type, mutated, and control) when a heterozygous status is present. Though ARMS PCR is a reliable method to detect point mutations in RTKs, its efficiency depends strongly on the region that needs to be amplified and the corresponding PCR design. Its sensitivity can reach up to the detection of 2.25% of point-mutated allele in a sample. At the moment, this method is not used in routine laboratory diagnosis of RTKs in AML.[80,81]

High-resolution melting analysis. High-resolution melting (HRM) analysis is a semi-quantitative method that is based on amplification of DNA fragments with a fluorescence dye enabling the generation of a melting profile of the DNA template. Mostly, the unspecific fluorescence dyes SYBR green or EVA green are used. These dyes incorporate into double-stranded DNA during the PCR. The generated fluorescence signal that is detected corresponds to the DNA amount in the sample. After amplification, which is performed similar to normal PCR, the products are denatured using HRM. The temperature is raised in 0.05 °C or smaller steps per second whereupon the change in the fluorescence signal is measured. When the melting temperature of the

amplified DNA fragment is reached, the DNA strands disso-
ciate from each other, leading to the release of the fluorescence
dye. This in turn induces a strong decrease in the fluorescence
signal. When the change of the fluorescence over time is
plotted against the temperature, a distinct melting profile is
generated. Depending on the base composition, the melting
temperature can increase or decrease allowing the differentia-
tion of point mutations, deletions, or insertions compared to a
wild-type allele.[82,83]

Tan et al used HRM analysis for the detection of *FLT3*-
TKD mutations.[84] Furthermore, Liu et al analyzed mutations
in childhood AML using this method.[85] *c-Kit* mutations were
detected by Willmore et al using HRM in gastrointestinal
tumors.[86]

Major advantages of this method are high throughput
of samples, rapidity, and convenience, making HRM a reli-
able presequencing screening method. Thus, only samples
lacking the possibility of an explicit interpretation need to be
sequenced. However, the data interpretation can be difficult.
Unexpected SNPs can interfere with the analyzed muta-
tion producing an imprecise shape of the melting profile.
Furthermore, because HRM relies on differences in the
melting temperature, the amplicon size needs to be as small
as possible so that the difference in the melting tempera-
ture is as high as possible. This allows a better differentiation
between mutant and wild-type allele. Due to this limitation,
HRM cannot detect mutations that encompass an entire
genome. In addition, different factors such as DNA concen-
tration and quality and magnesium chloride concentration
influence the melting profile of the DNA so that validation
of constant reaction settings has a great importance.[82,83,87]
RTK mutations in AML are mostly heterozygote, which
could result in a higher proportion of the wild-type allele
compared to the mutated allele. Sotlar et al described a
method to increase the sensitivity of HRM toward the
mutated allele using a peptide nucleic acid (PNA) for the
detection of *c-Kit* point mutations. These are mimics of DNA
in which the sugar phosphate backbone is replaced by a pseudo-
peptide. This structure ensures a high stability of PNAs and
generates a higher affinity to complementary DNA or RNA
sequences compared to analog DNA oligomers. Binding of
the PNA to the wild-type allele suppresses its amplifica-
tion and enables detection of a minor mutation fraction.[88]
Another possibility is the application of locked nucleic acids
(LNAs). These are modified RNA nucleotides in which the
ribose is fixed by an additional methylene bridge between the
2′-oxygen and the 4′-carbonate leading to a more inflex-
ible molecule. The characteristics that are generated are the
same as those for PNAs with an increased thermal stabil-
ity to complementary DNA compared to DNA oligomers.
The polymerase is not able to elongate DNA–LNA hybrids
leading to selective amplification of the mutated allele.
Warshawsky and Mularo reported that the usage of LNAs
enhanced the detection of *FLT3* mutations 10- to 50-fold.[89]

Lu et al described a probe-based melting analysis of *c-Kit*
mutations. Instead of an unspecific fluorescence dye, two probes
designated with different fluorochromes at their 5′-end are
used. One of them contains a 3′-quencher that attenuates the
signals of both fluorochromes. The assay was designed for the
detection of D816, D820, and N822. The application of spe-
cific probes could reduce the amount of factors that influence
the DNA melting profile when unspecific dyes are used.[90]

**Denaturing high-pressure liquid chromatography
analysis.** Denaturing high-pressure liquid chromatography
(DHPLC) is a method that is similar to HRM and relies
on the analysis of the melting profile of DNA. The DNA is
denatured at 95 °C and then cooled-down to room tempera-
ture, enabling renaturation. When a mutation is present, the
DNA forms heteroduplexes, whereas wild-type DNA forms
homoduplexes. These two forms can be separated by a high-
resolution polymer matrix using high-pressure liquid chroma-
tography. During further procedures, distinct melting profiles
are analyzed.[91]

DHPLC is applicable as a presequencing screening
tool because of high sensitivity and less subjective and labor-
intensive generation of data. However, the sequence composi-
tion and especially the flanking region near the mutation spot
(10–20 nucleotides) greatly influence the temperature that is
needed to detect heteroduplexes. Thus, a proper validation
of analysis temperature is essential. Because of the impact
of different factors on the detection of heteroduplexes, inter-
pretation of data is sometimes difficult. Furthermore, this
method is not well defined for the routine laboratory analysis
of RTKs.[92,93]

Real-time PCR (qPCR). In AML diagnosis, Real-Time
PCR (qPCR) is mainly used for the detection of minimal
residual disease (MRD), for example, when a *NPM1* insertion
mutation is present.[94] In contrast, the detection of point muta-
tions is more complicated because the reaction must have a
much higher sensitivity for the mutated allele. One possibility
is an allele-specific PCR with a blocking reagent, which uses
a blocker nucleotide against the wild-type allele. The blocker
contains a phosphate at its 3′-end, which inhibits amplifica-
tion of the wild-type allele by the PCR polymerase. This leads
to selective amplification of the mutated allele. However, the
validation of this method is time consuming because of the
high amount of reagents as primers, blocker, and probe that
need to be adjusted. The advantages are high sensitivity and a
fully quantitative method, which can be performed easy, fast,
and cheap when reliably established.[95–97]

Another problem of qPCR methods is the lack of com-
parability between different laboratories because of differences
in sample preparation and PCR performance. Variations are
also caused by using different real-time PCR machines.

FLT3-ITD can easily be detected by qPCR because it
harbors an insert similar to *NPM1* mutations.[98,99] It was dem-
onstrated that *FLT3*-ITD is a reliable marker for monitoring
the treatment response of patients with AML. But its role as a

marker for MRD is controversial. *FLT3* mutations show only low stability in the course of AML disease so that subclones with *FLT3* mutations can be lost while the patient falls into relapse. Another possibility is variation of the ITD leading to a false-negative result when patient specific primers are used. The generation of patient-specific primers is possible, but it is very expensive and time consuming and therefore not suitable for rapid routine laboratory diagnosis.[9,100–102]

The usage of unspecific fluorescence dyes as SYBR green for the quantitative mutation diagnosis is not recommended because unspecific products like primer dimers are also detected. In addition, qPCR methods exhibit different advantages and disadvantages that depend on the RTK which is amplified. This includes expression level, instability, and specificity of the RTK target.

Capillary electrophoresis (Gene Scan). The Gene Scan method is a standard PCR, which is performed with fluorescent-labeled primers in combination with a high-resolution electrophoretic separation. The separation mechanism is similar to agarose gel electrophoresis, but fragments are transported along a capillary and the size is measured by their fluorescence signal. Separation can be performed in a DNA sequencing machine. The main advantages of this method are rapidity, low cost, and high sensitivity up to 1%. Compared to HRM, analysis of bigger amplicon sizes is possible. This reduces the amount of primers needed and makes the method cheaper. In addition, it enables detection of minor mutant bands because of higher resolution compared to agarose gel electrophoresis. However, it is not suitable for detection of point mutations. For *FLT3*-ITD, a separation of a minimum difference of 3 bp was reported and the detection threshold was described between 0.8% and 2%. After endonuclease restriction, *FLT3*-D835 can also be analyzed with capillary electrophoresis.[103–105]

This method facilitates a semi-quantitative detection of the mutant allele. Thus, it is possible to calculate a mutational ratio using the peak area. By definition of distinct ratios that describe the mutational status of patients with AML, a standardized protocol for the patient report is possible. Bubán et al analyzed different electrophoretic methods for the detection of *FLT3* mutations. *FLT3*-ITD fragments were detectable at >330 bp, whereas the wild-type allele displayed a peak at 330 bp.[104,106]

DNA sequencing. Traditional DNA sequencing relies on the Sanger sequencing method, which was developed by Sanger et al in 1977.[107] First of all, the targeted DNA fragment is amplified by standard PCR. Then, a sequencing reaction is added using only one primer (forward or reverse). After denaturation of the DNA fragment, the primer anneals at a lower temperature, leading to primer extension and fluorescence labeling of the template. In each round of primer extension, the reaction is terminated by incorporation of fluorescently labeled dideoxynucleotide triphosphates (ddNTPs). Thereby the label on the terminating ddNTP corresponds to the nucleotide of the terminal position. Subsequent to the sequencing

reaction, redundant ddNTPs are removed by a cleaning step of the DNA template. In addition, shotgun de novo sequencing can be performed by cloning of randomly fragmented DNA into a high–copy number plasmid, which is then used to transform *Escherichia coli*.

The sequence is determined by high-resolution electrophoretic separation. Therefore, products are again denatured and then applied to a capillary-based polymer gel. The sequencing read-out is provided by a four-color detection of the emission spectra, which is generated by laser excitation of fluorescent-labeled fragments. When fragments of distinct length exit the capillary, the sequencing analysis software transforms the position of the respective fluorescence signal into a DNA sequence and also generates error probabilities for each base call.

The major advantage of DNA sequencing is that a large fragment of up to 1,000 bp can be analyzed. It can be performed almost for every target in part independent of the target sequence, and it shows a raw accuracy of more than 99.9%. This is of course due to the long period of time (around 30 years) in which the method was optimized. It is still seen as the gold-standard for mutation detection. However, it also exhibits disadvantages. The time and labor for this method are very extensive. In addition, Sanger sequencing shows limitations in detection sensitivity (only 10%–15%). Thus, low amounts of mutation alleles could be overlooked generating false-negative results.[108]

Sanger sequencing can be used to identify rare mutations in AML samples, for example, in *CSF1R* and *PDGFR α/β*.[71,109,110] Furthermore, Sanger sequencing is an important tool for the identification of specific mutations sites, for example, when analyzing *FLT3*-ITD mutations in the JMD and TKD.[66] When results achieved by previously described methods are unclear, sequencing can be used to enumerate and assure diagnosis. Also, Sanger sequencing is a valuable tool for the identification of heterogeneous mutations, for example, in *c-Kit* that could possibly influence AML pathogenesis.[111,112]

Next-generation sequencing. Since Sanger sequencing is a time-consuming method, the demand for a more rapid approach for the detection of mutations was present for a long time. About 8 years ago, the establishment of next-generation sequencing(NGS) has provided a platform for a great number of new applications for mutation detection.[113,114] Furthermore, NGS provided the possibility for high-throughput de novo sequencing of the human genome, MRD diagnostic, human leukocyte antigen compatibility testing, familiar genetic testing, and forensic DNA testing.

Massive parallel sequencing produces millions of short DNA fragment reads at the same time. The reads are assembled to larger sequence fragments (contigs) and mapped back to the reference genome, allowing discrimination of mutated and wild-type DNA.[115]

Different sequencing strategies are available. Whole-genome sequencing deciphers the complete genome. It requires

at least 30- to 40- fold haploid coverage of the genome to achieve a reliable diploid coverage for mutation detection. Whole-genome sequencing has the advantages that information about the complete genome is generated enabling identification of structural variants as translocations and deletions. However, data analysis is expensive and complicated. Because of the high read-depth that is needed for solid analysis, a high amount of mapping errors can be generated, leading to identification of around 50% false-positive sequence variants. Also, mapping to repetitive sequences is imperfect and can generate false-negative results. This accentuates the need for proper validation of NGS.[116,117] Most common NGS analysis software tools have been evaluated for their accuracy in detecting single-point mutations. In contrast, the detection of deletions and inserts is difficult so that *FLT3*-ITD analysis is problematic. Spencer et al described that only Pindel is able to reliably detect *FLT3*-ITD.[118]

Exome sequencing targets selectively 1%–2% of coding genes, microRNAs, and other noncoding RNAs. To achieve this selective sequencing, the genomic DNA is hybridized to beads containing probes that are designed to capture the 200,000 exons of the human genome. Because of the reduced amount of sequences, this method provides relative deep sequence coverage as well as reduced costs. But mutations outside exomes are not detected so that identification of structural variants is not possible.[119,120] Using this method, Opatz et al identified a recurring *FLT3* N676K mutation in CBF leukemia.[121] Furthermore, Guerreiro et al analyzed mutations of *CSF1R* in inherited leukodystrophies using exome sequencing.[109] Because activating mutations of *CSF1R* are rare in AML, NGS can enable the detection of infrequent mutations, possibly contributing to disease pathogenesis.

Transcriptome strategies sequence all transcribed genes as well as coding and noncoding RNAs. The major advantage of this method is that information about quantitative gene expression levels, posttranscriptional changes, and fusion transcripts are detected. However, transcriptome sequencing does not detect mutations in noncoding regions and thus also no mutations that induce RNA turnover (like frameshift and nonsense mutations) or cause the loss of one or both copies of a gene. Because the amount of transcribed genes is dependent on their expression strength, this sequencing strategy is biased toward abundantly expressed genes, causing difficulties in the analysis of genes with low or absent gene expression.[117,122] Greif et al identified tumor-specific somatic mutations in AML using this method.[123]

When NGS is solidly validated, targeted application can provide comprehensive and unbiased mutational profiling of many genes at the same time. The usage of one single method would reduce the costs for mutation detection and minimize the experimental labor. The increased sensitivity of NGS (1%–2%) is an additional advantage compared to Sanger sequencing. However, the actual detection limit depends on the portion of errors generated during the sequencing process.

Thereby, errors can arise during library construction and sequencing, for example, by polymerase errors.

Currently, NGS remains expensive and the required bioinformatics know-how and time to perform data analysis hinder rapid routine diagnostic applications. Methodological differences in the commercially available NGS platforms as template preparation, sequencing, imaging, and data analysis additionally complicate the introduction of generalized diagnostic procedures. Depending on the NGS platform used, the quantity of errors can range up to several percent. Further mistakes can be introduced by data analysis when the received sequence variants are mapped against reference data from public databases. In addition, this process can be performed with various software packages and different base-calling algorithms.[124,125]

When analyzing mutations in RTKs, it is important to compare a malignant patient's sample to a healthy donor. Humans harbor three to four millions of inherited sequence variants so that the majority of detected sequence differences are inherited single-nucleotide polymorphisms and not acquired mutations. Furthermore, the huge amount of mutational spots found in samples of patients with AML indicate that most of these variations are secondary events and do not contribute to leukemic transformation. Therefore, it is difficult to identify "driver" mutations from the generated massive data sets.[126,127] Loriaux et al analyzed the tyrosine kinome using NGS and found a high amount of sequence variants that had no influence on the RTK activity and thus no functional significance for AML.[128]

In lymphatic disorders such as acute lymphoblastic leukemia (ALL) and lymphoma, MRD diagnostic was successfully performed using NGS. NGS was validated for this purpose because previously available allele-specific oligonucleotide (ASO) PCR could only be performed with patient-specific primers. Using NGS amplification of IgH genes with universal primers high throughput sequencing for disease-specific IgH sequences was enabled.[129–131] Luthra et al evaluated the MiSeq sequencing platform for mutational screening in patients with AML. They found that MiSeq is highly applicable for diagnostic approaches because of its simplicity, high specificity, and accuracy.[132]

An optimal but distant approach would be to combine NGS with real-time PCR and microarray analysis. This technique would allow estimation of gene expression signature, hematopoietic lineage, and chromosomal rearrangements or translocations of leukemic blasts.[124] At this point, reliability of NGS can be hiked by combination with capture methods or molecular barcodes. Using capture methods, a specific region of interest that is important for prognosis and therapy is enriched using hybrid capture, multiplex PCR, or ligation-mediated methods. Hybrid capture covers a comparatively big genomic region and has a high flexibility but low on-target efficiency. In contrast, PCR methods enrich a smaller genomic part but exhibit higher on-target efficiency

and faster turn-around time.[133] Molecular barcodes are short, degenerated oligonucleotides that can be used to trace specific DNA sequences. The barcodes are added during enrichment or sequencing. Generated sequencing data are verified with the barcode, whereby all duplicate reads with the same barcode need to contain the same sequence variants. This enables reduction of sequencing errors and false-positive results. With respect to limitations in DNA quantity, the sensitivity is approximately 1 mutated cell in 2,500 normal cells.[133,134]

Marker panels in combination with amplicon deep-sequencing enable analysis with high sensitivity and fast patient throughput, which could be useful in clinical studies for monitoring of treatment response.[124] Additionally, NGS-based assays facilitate increased detection sensitivity of small clonal populations. Thus, evolution of leukemic blasts in the course of therapy could be identified, generating new insights about acquired resistance mechanisms. This could greatly improve documentation and testing of new therapeutics in clinical studies. Moreover, improvement of the reliability of clinical study data can be achieved by combination with high-sensitivity MRD testing using NGS as described for ALL. However, application of NGS in clinical studies requires carefully considered informed consent, for example, when mutations with clinical implication for the patients children are identified.[119]

NGS provides the possibility to detect new correlations between mutations in RTKs and downstream effectors possibly leading to the discovery of new pathways responsible for resistance generation. In addition, sample analysis at different time points of AML disease using NGS supports new insights about the clonal selection and evolution of leukemic blasts, leading to differentiation between early AML triggering and secondary acquired mutations.[135]

Due to the above-mentioned difficulties in validation and generalization of NGS, it will take several more years until this method can be reliably applied in the routine diagnostic of RTK mutations in patients with AML. When these barriers can be overcome, NGS would greatly improve the daily routine for diagnostic mutation detection in various diseases.

Conclusion

Mutations in RTKs are common and well defined in patients with AML. Insertions and point mutations lead to constitutive activation of RTKs, triggering uncontrolled proliferation of leukemic blasts. Because their presence has a major influence on prognosis and treatment of patients with AML, their reliable detection in routine laboratory analysis is very important. PCR-based methods are used to identify the most common mutations in *FLT3* (D835 or ITDs) and in *c–Kit* (D816). These methods benefit from a fast, reliable, and cheap application but cannot identify rare mutations. Using melting analysis such as HRM and DHPLC, mutation detection in AML samples can be expanded toward a semi-quantitative approach and identification of infrequent mutations. However,

data interpretation can be difficult. Gene Scan methods also generate semi-quantitative data and are easier to analyze. Fully quantitative mutation detection can be enabled using real-time PCR, but with this method, only small amplicon sizes can be analyzed. Sequencing-based methods such as Sanger sequencing and NGS are almost completely independent of sequence composition, permitting identification of rare and heterogeneous mutations, although Sanger sequencing can be cost and time intensive. Furthermore, NGS is an emerging method with room for improvement regarding costs and data analysis. All applicable methods have their advantages and disadvantages depending on the target and detection aim. A robust combination of different methods can provide a solid generation of data and interpretation. At the moment, NGS methods are difficult to validate for routine laboratory diagnosis. Until the accuracy of NGS has improved and standardized operating procedures and sequencing panels are established, Sanger sequencing will remain the method of choice for sequencing diagnostic approaches.

Author Contributions
Wrote the first draft of the manuscript: RB. The author reviewed and approved of the final manuscript.

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