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Review Article

Myeloid Neoplasms: Better Understanding of their Molecular Pathogenesis with Improvised Genomic Testing: A Ray of Hope for Better Clinical Outcomes

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Abstract

With the increase in incidence and prevalence of myeloid neoplasms in India, it has become a necessity to understand its molecular mechanisms, acquisition of genomic alterations, and understand its primary and secondary resistance pathways which ultimately impact the decision of therapeutics. The objective of this review is to investigate the molecular aspects of this disease type and identify the biomarkers that help with diagnosis, risk assessment, prognosis, and selecting the best line of treatment for a specific myeloid neoplasm. Advancements and innovations in molecular technologies from simplest Real-Time PCR to high throughput next-generation sequencing have played a vital role in screening the most common mutations and fusions to the novel and rare. Molecular technologies have helped to enumerate the genomic landscape of myeloid malignancies. The understanding of both- the mechanisms and the technology is a strong combination as it has helped revolutionize precision oncology and helped in giving better therapeutic choices with better clinical outcomes. The importance of cellular morphology, clinical symptoms, and molecular pathology in assessing the risk of myeloid malignancies is emphasized and summarized in the review. The review concludes that understanding molecular pathogenesis can be improved by using clinical-pathological-molecular strategies for diagnosis and therapy decision-making.

More Information

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Introduction

In the United States, leukemia ranks eleventh among cancer types while in India it precedes to rank third generating alarm in view of its prevalence and incidence [1] (The Leukemia & Lymphoma Society). All cells which are granulocytes (eg. neutrophil, eosinophil, and basophil), monocyte/macrophage, erythroid, megakaryocyte, and mast cell lineages are collectively referred to as "myeloid." Myeloid malignancies are clonal disorders of the progenitor cells or Hematopoietic Stem Cells (HSCs). They are divided into acute stages, such as acute myeloid leukemia (AML), and chronic phases, such as myeloproliferative neoplasms, myelodysplastic disorders, and chronic myelomonocytic leukemia [2]. These are caused by genetic and epigenetic changes that interfere with important functions like cellular differentiation, cellular self-renewal, and cellular proliferation.

The World Health Organization (WHO) has released its latest version i.e. Fifth edition of the WHO classification of hematolymphoid tumors in the year 2022 (August) [3]. Since its last release in 2008 and revision in 2017, the World Health Organization (WHO), the Society for Hematopathology (SH), and the European Association for Haematopathology (EAHP) have worked together to create a comprehensive and unambiguous classification of neoplasms of hematopoietic and lymphoid tissue into two categories: myeloid and lymphoid to have unanimous terminology and segregation of leukemia type. This classification is based on feedback from Clinical Advisory Committees (CACs), which are made up of a panel comprising pathologists, hematologists, oncologists,

and geneticists. Comprehending the mutation landscape and forecasting its clinical prognosis, has provided molecular pathologists, medical oncologists, and clinicians with ground-breaking guidance in developing accurate diagnosis, risk assessment, and selecting appropriate therapeutic decisions. It has also opened the broad horizon of understanding the mutational profiling of a particular leukemia type by understanding the affected molecular pathway which has resulted in leukemogenesis and also helped to anticipate resistance mechanisms [4].

Myeloid neoplasms and their mechanisms [5]

WHO (2022) has classified myeloid neoplasms into nine types:

1. Myeloid precursor lesions,
2. Myeloproliferative neoplasms (MPNs),
3. Mastocytosis,
4. Myelodysplastic neoplasms (MDNs, previously known as myelodysplastic syndrome, MDS),
5. MDN/MPNs,
6. Acute myeloid leukemia (AML),
7. Secondary myeloid neoplasms,
8. Myeloid/lymphoid neoplasms with eosinophilia and defining gene rearrangement,
9. Acute leukemias of mixed or ambiguous lineage.

Further sub classified into: The classification has helped to categorize the patient into the right leukemia type by co-relating clinical presentation, cellular morphology, immunophenotype, and genomic alterations giving a holistic picture of the patient's diagnosis, risk, prognosis, and therapeutic response (Table 1).

Physiology: A balanced approach: Hemostatic equilibrium between all lineages of blood cells produced helps to maintain self-renewal, quiescence, and differentiation of HSCs. This equilibrium may be affected by aging (this process has detrimental effects on HSCs and the hematopoietic system) and stress [6].

Pathology: The skewed homeostatic equilibrium leads to an imbalance between different cell lineages leading to the accumulation of heritable or somatic gene mutations and the development of a heterogenous group of diseases characterized by the dysfunctional production of myeloid cells in bone marrow.

Molecular pathology: The presence of accumulation of driver mutations leads to preleukemia and progress to leukemia conditions assisted by latency and evolutions of mutational landscape. Also, there are presence of different subclones leading to further accumulations and forming complex tumor structures which creates a mosaic of cellular clones with the presence of a unique set of mutations, hence co-occurrence of various gene mutations and fusions are evidenced. These discoveries have now been made possible by broad molecular profiling, which also aids in their selection of therapies. Also, studying these subclones helps us to understand resistance mechanisms to any target therapy or chemotherapy along with anticipated drug toxicities. Various cellular mechanisms: Transcriptional regulation, epigenetics (modifications of DNA and histones),

Table 1: Classification of Myeloid Neoplasms [5].

1	Premalignant clonal cytopenias and myelodysplastic syndromes Clonal cytopenia of undetermined significance Myelodysplastic syndrome with mutated SF3B1 Myelodysplastic syndrome with del(5q) Myelodysplastic syndrome with mutated TP53 Myelodysplastic syndrome, not otherwise specified (MDS, NOS) MDS, NOS without dysplasia MDS, NOS with single lineage dysplasia MDS, NOS with multilineage dysplasia Myelodysplastic syndrome with excess blasts Myelodysplastic syndrome/acute myeloid leukemia (MDS/AML) MDS/AML with mutated TP53 MDS/AML with myelodysplasia-related gene mutations MDS/AML with myelodysplasia-related cytogenetic abnormalities MDS/AML, not otherwise specified
2	MPNs Chronic myeloid leukemia Polycythemia vera Essential thrombocythemia Primary myelofibrosis Early/pre fibrotic primary myelofibrosis Overt primary myelofibrosis Chronic neutrophilic leukemia Chronic eosinophilic leukemia, not otherwise specified MPN, unclassifiable
3	Mastocytosis
4	Myelodysplastic/myeloproliferative neoplasms Chronic myelomonocytic leukemia Clonal cytopenia with monocytosis of undetermined significance Clonal monocytosis of undetermined significance Atypical chronic myeloid leukemia Myelodysplastic/myeloproliferative neoplasm with thrombocytosis and SF3B1 mutation Myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis, not otherwise specified Myelodysplastic/myeloproliferative neoplasm, not otherwise specified
5	Acute myeloid leukemias
6	Myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase gene fusions Myeloid/lymphoid neoplasm with PDGFRA rearrangement Myeloid/lymphoid neoplasm with PDGFRB rearrangement Myeloid/lymphoid neoplasm with FGFR1 rearrangement Myeloid/lymphoid neoplasm with JAK2 rearrangement Myeloid/lymphoid neoplasm with FLT3 rearrangement Myeloid/lymphoid neoplasm with ETV6::ABL1
7	Pediatric and/or germline mutation-associated disorders Juvenile myelomonocytic leukemia Juvenile myelomonocytic leukemia-like neoplasms Noonan syndrome-associated myeloproliferative disorder Refractory cytopenia of childhood Hematologic neoplasms with germline predisposition
8	Acute leukemia of ambiguous lineage Acute undifferentiated leukemia Mixed phenotype acute leukemia (MPAL) with t(9;22)(q34.1;q11.2); BCR::ABL1 MPAL, with t(v;11q23.3); KMT2A rearranged MPAL, B/myeloid, NOS MPAL, T/myeloid, NOS
9	Secondary myeloid neoplasms

DNA damage signaling and repair, chromosome segregation and replication (cohesin complex), RNA splicing, and signal transduction are disrupted in leukemia and lead to its development. Transcription factors, RNA splicing proteins, and deubiquitinating enzymes are among the numerous processes that are gaining popularity in therapeutic interventions [7-9].

Pathogenesis

Cytogenetics: Karyotype and Fluorescence *in situ* Hybridization (FISH) are established as the gold standard in hematological malignancies however, they don't always enable low clonal cell detection due to their technical sensitivity and lower limit of detection constrain [10,11]. In addition to highlighting the significance of cytogenetics, the WHO and European Leukemia Net (ELN) in the International Consensus Classification (ICC) in 2022 have highlighted the importance of somatic mutations (driver mutations) by molecular testing. This review uses this viewpoint as inspiration to explore the molecular landscape across various myeloid neoplasms. Myeloid malignancies are always popular with their clinical features, morphological features, immunophenotypes, cytogenetic and functional pathobiology. There has been little research on the genetic landscape with respect to patient prognosis, treatment difficulties, and overall survival [12-15].

Cellular pathways in myeloid neoplasms

It's immensely essential to characterize a certain leukemia type based on the cellular pathways affected. This will assist in identifying the greatest number of potential biomarkers and separating them into categories such as diagnostic, prognostic, predictive, or biomarkers that have the potential to lead to therapeutic resistance. The aim of this approach is solely for better clinical outcomes, the most precise therapeutics choice, and risk assessment (Table 2) [16].

Clonal hematopoiesis of indeterminate potential (CHIP) in myeloid malignancies

A subtype of clonal hematopoiesis is known as Clonal Hematopoiesis of Indeterminate Potential (CHIP), which is characterized by point mutations and minor insertions and deletions in recognized leukemia-driver genes like DNMT3A, ASXL1, TP53, JAK2, SRSF1, SRSF2 or TET2. The enhanced ability to self-renewal in preleukemia HSCs results in clonal growth of HSCs because of the presence of recurrent somatic mutations without signs of dysplasia, cytopenia, or malignancy. The aging process is strongly correlated with the presence of CHIP. According to recent studies, people over 70 years old have 10% CHIP, and people over 90 years old have 20% CHIP which is termed age-related clonal hematopoiesis (ARCH). Compared to older individuals, their occurrence is significantly lower in those under 50, which increases the chance of neoplasia development when they are present. Hence while considering [17-19]. In 2019, a clinical investigation was carried out on 81 patients who were suitable for Autologous Stem Cell Transplantation (ASCT) and had solid tumors or lymphoid disorders. The study focused on screening the development of CHIP mutations. A 22% incidence of CHIP was noted, and the highest mean variant allele frequency was found to be 10.7%. Based on the study's findings, the authors concluded that there is a higher chance of CHIP-related issues in the future since CHIP-mutated stem and progenitor cells essentially expand in size during ASCT-related blood reconstitution [20].

Molecular technologies and their advancements in combating myeloid neoplasms

Recent years have seen a rise in the popularity of a variety of molecular technologies, from the quick, lesser cost, less complex real-time PCR, karyotyping, and FISH techniques to the extremely complex Sanger sequencing, and next-generation sequencing with targeted panels, clinical exome sequencing, and whole exome sequencing which drawback of cost and more Turn Around Time (TAT). Patients now have a better chance of receiving the best treatments available for their leukemias because of the advancements in biotechnology in India and the availability of several gene panels. The era of Precision Oncology has revolutionized the treatment opportunities offering benefits in terms of improvised progression-free survival and overall survival.

Molecular testing with extracted DNA and RNA from the patient's blood and bone marrow has given the opportunity to study and decipher the mutational landscape comprising of short nucleotide variants, insertions, deletions, and gene fusions which further helps to sub-characterize myeloid neoplasms. Owing to innovations in molecular technologies, it is now possible to establish large mutational databases that include biomarkers for myeloid neoplasms that are: a) predictive, b) prognostic, and c) diagnostic.

The following recurrent genetic anomalies are used by the WHO classification to identify different subtypes of myeloid malignancies: t(8;21)(q22;q22) (RUNX1-RUNX1T1), t(9;22)(q34;q11) (BCR-ABL1), inv(16)(p13.1q22) or t(16;16)(p13.1;q22) (CBFB-MYH11), t(15;17)(q24;q12) (PML-RARA), t(9;11)(p22;q23) (MLL-MLLT3), t(6;9)(p23;q34) (DEK–NUP214), inv(3)(q21q26.2) or t(3;3)(q21;q26.2) (RPN1–MECOM), and t(1;22)(p13;q13) (RBM15-MKL1).

Real-time Polymerase Chain Reaction (RT-PCR)

With its innovative advancements, real-time polymerase chain reaction (RT-PCR) has emerged as a rapid screening tool for prevalent gene mutations and fusions in basic multiplex panels, particularly in cases that have recently been diagnosed. RT PCR developed in 1984 by Karl Mullis, amplifies a target DNA molecule and produces detection curves. These detection curves are divided into four phases:

Table 2: Cellular Pathways and Genes Affected Leading to Diagnostic and Prognostic Implication in Myeloid Neoplasms.

Transcriptional regulation	Function: Cell maintenance, differentiation, and maturity are all controlled by transcription factors. Genetic Impact: The presence of somatic mutations, translocations, and aberrant expressions can lead to malignant transformation of hematopoietic cells and encourage tumorigenesis. formerly, these components were deemed "not druggable" due to their lack of enzymatic activity. However, new focused therapeutic approaches have been created recently with an understanding of the genetic and epigenetic principles of transcription factor control [33].	ELANE, MPO, BCOR, BCOR1, CDKN2A, CEBPA, CSF3R, ETV6, GATA1, GATA2, MYC, PHF6, RUNX1, SMAD9/9L
Epigenetics (modifications of DNA and histones)	Function: Lineage differentiation, and self-renewal by regulating transcriptional factors. Genetic Impact: The presence of somatic mutations, and gene aberrations promote and inhibit transcriptional activity without changing the underlying DNA sequences. Any imbalance in this regulation (at the sites of promoter or enhancer) leads to tumorigenesis. Many novel targeted therapies are developed that prevent relapse and improve long-term survival [34] (Xuemeng Xu 2023).	EZH2, PRC2, H3K27me3, ASXL1, DNMT3A, EED, EP300, EXH2, IDH1, IDH2, TET2
DNA damage signaling and repair	Function: vital function in shielding cells from external or endogenous factors that can cause varied levels of DNA damage when they are repeatedly exposed to them Genetic Impact: The decrease in DNA repair mechanisms may lead to an increase in the number of clones. In order to combat malignancy, checkpoint inhibitors cause genetic instability in cancer cells, which has greatly increased therapy response and survival rate. Concurrent inhibition of DNA repair pathways can somewhat attenuate the development of resistance to DNA-damaging chemotherapeutic drugs including cisplatin, cyclophosphamide, chlorambucil, and temozolomide while enhancing cytotoxicity [35-37].	BRAF, BRCC3, NPM1, PPM1D, SETBP1, TP53, WT1
chromosome segregation and replication (cohesin complex),	Function: Unique ring structure. Vital role in chromosome segregation, DNA replication, DNA damage response, and transcriptional regulation through chromatin looping. Cohesin can both facilitate as well as antagonize PcG (PRC1/PRC2)-mediated chromatin interactions, depending on the genomic site and cell context. Genetic Impact: Loss of STAG2 has been associated with defects in the replication process in the form of halted and asymmetric replication fork. Cell division and its regulation are important for maintaining the ploidy of the cell and failure to do so results in aneuploidy, which has been closely associated with cancer Inhibitors of cohesin regulators Aurora kinase B, polo-like-kinase 1, cyclin-dependent kinase 1 induce cell death in cancer and some others are currently in clinical trials (Minchell, et al. 2020).	ATRX, PDS5B, RAD21, SMC1A, SMC3, STAG1, STAG2
RNA Splicing Regulation	Function: Regulatory step in proper control of gene expression. complex and highly regulated process involving the removal of introns and the ligation of exons to produce mature mRNAs for protein translation Genetic Impact: Recent work has highlighted alternative splicing as a mechanism of resistance to chimeric antigen receptor-expressing T-cell therapy (CART). Given the importance of alternative splicing dysregulation in cancer initiation and progression, there has been significant interest in developing therapeutic strategies to target aberrant splicing in cancer. Various therapeutic modalities have been proposed and are at different stages of pre-clinical and clinical development ranging from small molecules [38,39].	DDX41, PRPF8, SF3A1, SF3B1, SRSF2, U2AF1, U2AF2, ZRSR2
Signal transduction	Function: p21ras pathway, c-myc pathway, and Jak-STAT pathway Genetic Impact: mutation or epigenetic alteration in hematopoietic stem cells (HSC), leads to the generation of the pro-inflammatory milieu in the marrow microenvironment that can result in apoptotic cell death of normal HSCs. Inhibition of myelosuppressive cytokine signaling cascades can stimulate hematopoietic activity in HSCs. Currently, P38 MAPK inhibitors, mTOR inhibitors, TGF-β pathway inhibitors, MEK inhibitors, and a few other compounds are being tested in various stages of clinical development. Finding an appropriate combination of novel agents and dosing frequencies that will enhance hematologic recovery would remain a challenge that needs to be addressed in newer studies. Future studies will be aided by correlative studies of Gene mutations, aberrant DNA cytosine methylation, and other genetic/epi- genetic biomarkers that will help identify a subset of MDS patients who might respond well to these new agents [40,41](Krenn & Aberger 2023).	PICAM, ALK, BRAF, CBL, FLT3, JAK2, KIT, PDGFRA, PTPN11, NF1, NRAS, KRAS,

(i) Linear ground, (ii) early exponential, (iii) log-linear (iv) plateau. The information acquired during these stages is crucial for determining cycle threshold, background noise, or amplification efficacy, and for assessing it both quantitatively and qualitatively for diagnostic and monitoring purposes in myeloid malignancies. Technology has become more popular as it assists in making prompt therapeutic decisions in response to identified reports. Though it allows us to have better turnaround time (TAT) at an affordable cost it doesn't allow us to screen a high number of gene mutations in one go or at multiple positions [21,22]. Also, the main drawback of RT-PCR is that it detects only DNA templates along with nonspecific binding (sybr green). A special enzyme – called Reverse Transcriptase – converts RNA into DNA templates, also known as complementary DNA (cDNA) [23]. It can be challenging to make decisions when an undetected report is received, and in rare cases with acute promyelocytic leukemia (APML), a chance can occur where the partner gene that differs from RARA or may have a three-way translocation with a false negative report as the approach for RT PCR panels are targeted. If treatment is not promptly administered, the patient will not survive. Karyotype and FISH thus play a significant influence in these circumstances holding that cost is the concern.

Advantages: Quick screening of target mutations of clinical interest and benefits, Faster TAT, Affordable cost.

Karyotyping

Chromosome sorting and identification, or karyotyping, is a commonly used technique to decipher normal or complex karyotype (poor prognosis) in myeloid malignancies and has gained popularity since the 1950s. The prognostic classifications provided by the UK Medical Research Council recommendation and the European Leukemia Net classification differ in terms of the number of single

aberrations and the notion of imbalanced aberrations. This is determined by chromosomal shape and gene location alterations. Karyotyping gives you the liberty to screen translocation, deletion, inversion, mosaicism, and chimerism which assist in the diagnosis and risk stratification of myeloid malignancies. While discussing the sensitivity of the technology, it finds both large and small numerical structural chromosomal aberrations (structural abnormalities of ≥ 3 - 5 Mb of DNA). The primary drawback of traditional karyotyping is its incapacity to reliably detect complicated chromosomal markers and cryptic abnormalities. In the chromosomal banding technique if the quality of metaphase is inferior, high-resolution analysis cannot be performed. Hence, the chances of missing out on the structural abnormalities are high. FISH has better performance characteristics in diagnosing myeloid malignancies [4,24,25].

Advantage: Gold Standard for diagnosis, ability to detect complex karyotypes.

Fluorescent *in situ* hybridization (FISH)

To identify chromosomal abnormalities, a technique known as fluorescent *in situ* hybridization (FISH) uses fluorescein-labeled DNA probes to hybridize to particular chromosomal areas. Highly sensitive to the detection of small genetic alterations, required technical expertise to diagnose the results. The abnormalities detected by this technique are translocations, deletions, inversions, trisomy, or amplification. The power of these probes results from the fact that analysis can be performed on interphase nuclei, facilitating the analysis of many more cells and providing details concerning percentages of cells that are positive or negative for the rearrangement. Cytogenetic and FISH Techniques in Myeloid Malignancies Fluorescence *in situ* hybridization (FISH) complements metaphase cytogenetics by the ability to evaluate large numbers of both interphase and metaphase nuclei. FISH is known to give rapid detection of specific translocation, gene amplification/copy number gain, assess minimal residual disease in myeloid malignancies, and also help to confirm the rearrangements that are difficult to conclude in karyotypes. Limitations with this technique while diagnosis and risk stratifying myeloid malignancies are labor intensive, time-consuming, chances of false negative if the specimen submitted for testing has sample integrity issues, the presence of cell fixation artifacts, and requires expensive instrumentations [26-28].

Advantage: Better sensitivity, Accurate results, Minimal Residual Disease (MRD).

Sanger sequencing

Sanger sequencing invented by Frederick Sanger and his team in 1977 is well-known for its chain termination technique. This is a DNA sequencing-based methodology that specifically addresses the role that DNA polymerase plays in chain-terminating deoxynucleotides during DNA replication. This method's primary benefit is its ability to recognize legitimate genomic events and other technology errors. The primary drawback of this method is its sensitivity, which accounts for 15% - 20% of the variant allele burden. This means that uncommon or novel mutations may go unreported. Additionally, the method is inadequate for screening very long DNA specimens and becomes more time-consuming, costly, and labor-intensive when several targets must be tested [29,30].

Advantage: Confirmation of genomic events and elimination of technology errors.

Next generation sequencing

Also, known as massively parallel signature sequencing, was first launched by Lynx Therapeutics Company in the year 2000 [31]. It is a high throughput technique that allows thousands and millions of short DNA fragments to be sequenced in one go in less time and is comparatively cost-effective when we target the same amount of data into other technologies. It is popular in sequencing whole genomes, whole exomes, and target genes. We can screen various cellular pathways affected along with resistance mechanisms that are present can be screened well in advance to avoid any therapeutic failures. Higher sensitivity and can detect up to 10^{-6} variant allele burden. This is more commonly used in established cases to monitor the measurable residual disease and to confirm if the patient has achieved a complete response to therapeutics. The technique also helps to detect various gene mutations, splice site mutations, insertions, deletions, and gene fusions. And also, understand the role of CHIP mutations which are explained in this review. NGS is a highly reliable tool, however, with advancements comes challenges which are the presence of sequencing artifacts that can be encountered in library preparation. Pipelines need to be extensively validated to detect the variants. Highly complex techniques, molecular pathologists, and scientists need to be highly trained to interpret the mutations and their relevance in leukemogenesis [29,32].

Advantage: Best sensitivity to detect up to 10^{-6} Variant Allele Frequency (VAF), broad screening of multiple biomarkers in one go, Minimal Residual Disease (MRD), screen cellular pathways and resistance mechanisms, scalability.

In recent times in India, following COVID 19 Pandemic, molecular technologies like RT PCR, NGS based panels like Whole Genome Sequencing, Whole Exome Sequencing, and Target Panel Sequencing have gained popularity and have generated awareness amongst clinicians, pathologists, scientists, and patients on the useful information which we can gathered. It will be easier to utilize the expertise and understanding and apply it to hematological (myeloid) malignancies. Further workups are required in advancing

molecular testing, especially the capacity of sequencing with more patient data generation at a lesser cost. Myeloid neoplasms' still have a poor prognosis. This will be improvised with the use of therapies that target pathways essential to leukemia cells' survival and leukemic stem cells. This needs to be done in conjunction with a highly focused strategy. Larger cohort studies are required and warranted, particularly in all forms of leukemia (chronic or acute), to comprehend the molecular pathogenesis and develop predictive models incorporating different genomic abnormalities that are encountered: gene fusions and mutations. To comprehend its prognosis, Copy Number Variants (CNVs) must also be added [42-51].

Conclusion

Understanding the altered cellular pathways in myeloid malignancies is essential since these alterations promote survival and proliferation. Targeted therapies are clinically beneficial in the precision oncology era since they have improved the overall survival rate and progression-free survival rate. To improve clinical outcomes and therapeutic response, it is imperative to administer the appropriate treatment at the appropriate time. Gaining knowledge of resistance mechanisms helps patients receive more effective treatment. With the use of advanced and quick molecular testing techniques like RT PCR, FISH, Karyotype, NGS, etc., all these needs can now be met. The review provides a means of establishing connections between the mutational profile, cellular morphology, and clinical presentation.

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