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Original article

Cytogenetics in the management of hematological malignancies: An overview of alternative technologies for cytogenetic characterization

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ABSTRACT

Genomic characterization is an essential part of the clinical management of hematological malignancies for diagnostic, prognostic and therapeutic purposes. Although CBA and FISH are still the gold standard in hematology for the detection of CNA and SV, some alternative technologies are intended to complement their deficiencies or even replace them in the more or less near future. In this article, we provide a technological overview of these alternatives. CMA is the historical and well established technique for the high-resolution detection of CNA. For SV detection, there are emerging techniques based on the study of chromatin conformation and more established ones such as RTMLPA for the detection of fusion transcripts and RNA-seq to reveal the molecular consequences of SV. Comprehensive techniques that detect both CNA and SV are the most interesting because they provide all the information in a single examination. Among these, OGM is a promising emerging higher-solution technique that offers a complete solution at a contained cost, at the expense of a relatively low throughput per machine. WGS remains the most adaptable solution, with long-read approaches enabling very high-resolution detection of CAs, but requiring a heavy bioinformatics installation and at a still high cost. However, the development of high-resolution genome-wide detection techniques for CAs allows for a much better description of chromoanagenesis. Therefore, we have included in this review an update on the various existing mechanisms and their consequences and implications, especially prognostic, in hematological malignancies.

Introduction

The *Groupe Francophone de Cytogénétique Hématologique* (GFCH), in its new version of hematological cytogenetics guidelines, proposes an update, within a set of 13 articles, of the abnormalities to look for in order to optimize the therapeutic management of patients suffering from hematologic disorders. The other articles in these recommendations are evidence of the constant involvement of Cytogenetics for the diagnosis and prognosis of hematologic malignancies. This is confirmed by the latest versions of the World Health Organization, the International Consensus Classification Committee and the European Leukemia Net (WHO-HAEM5, ICC, ELN) classifications [1–5], in which chromosomal abnormalities (CAs) play an important role. Chromosome banding analysis (CBA) and fluorescent in situ hybridization (FISH) remain the reference methods in most laboratories, following international recommendations. In many hematological malignancies, most prognostic scores are based on CAs, with an increasing proportion of molecular abnormalities, as well as copy number alterations (CNAs) and structural rearrangements (SVs), not seen with CBA and FISH. Thus, the emergence of pangenomic or targeted high-resolution technologies are about to disrupt the established practices of cytogenetic laboratories. Akkari et al. [6] recently published an interesting analysis of the current state of cytogenetics in hematologic malignancies and attempted to forecast the future opportunities and advances in our field. This article details the limits to near-term progress in cytogenetics, while suggesting that whole genome sequencing (WGS) is the main technique having the potential to

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replace the gold standard cytogenetic techniques. Here we present an addendum to the recommendations of the GFCH: an overview of alternative and complementary techniques to CBA and FISH that we think will be of technical interest to cytogeneticists (Table 1). Our selection is not intended to be an exhaustive list of current technologies, but we focused on techniques that are the most effective to detect SVs and/or CNAs that we believe are readily available to cytogenetic laboratories. As innovative technologies continue to blur the line between chromosomal and molecular genetic laboratories, we have included techniques commonly used in molecular genetic laboratories to detect SVs and CNAs. Some of these techniques have been used routinely for several years. Chromosomal microarray analysis (CMA) and multiplex ligation-dependent probe amplification (MLPA), being excellent complementary techniques to gold standard cytogenetic techniques, are widely used in cytogenetic laboratories but inconsistently used for hematology indications. RNA-sequencing and ligation-dependent RT-PCR (LD-RTPCR) reveal the consequences at the transcript level of some chromosomal abnormalities. Optical genome mapping (OGM) and long-read WGS (LR-WGS) technologies appear promising for replacing the gold standard tests. Although chromatin conformation analysis is not widely used in routine cytogenetic laboratories, it could provide information on the presence of structural rearrangements, particularly those involving immunoglobulin (IG) genes in lymphomas and can be applied to FFPE samples. This review will explain these techniques, their advantages and disadvantages from the cytogeneticist's perspective, and how they may be useful in the laboratory. We present these techniques

Table 1
Characteristics of alternative technology and the spectrum of detectable/non-detectable chromosomal abnormalities.

	CBA	FISH	CMA	MLPA	OGM	LD-RTPCR	Chromatin conformation analysis	LR-WGS	RNA-seq
Technical considerations									
Matrix	Chromosomes in dividing cells	DNA in interphase nuclei and metaphase	DNA	DNA	DNA	RNA	DNA	DNA	RNA
Prior cell culture	☑	possible	possible	☑	possible	☑	☑	possible	☑
Cytogenetic pellet extraction	NA	NA	☑	☑	under development	NA	NA	under development	NA
DNA quality	NA	NA	high quality	high quality	UHMW (> 150kb)	NA	all (FFPE included)	high quality	NA
Coverage	whole	targeted	whole	targeted	whole	targeted	whole (Hi-C) and targeted (FFPE-TLC)	whole	whole or targeted
Resolution	5-10 Mb	150 kb	30 kb	1b to 80 Mb	> 500pb or > 5kb depending on pipeline	100-1000 bp	up to 35kb in Hi-C	SNV	gene level
Sensibility	1-3 out of 20 metaphases	1-5%	25-30%	25-30%	5~10% SV / 20% CNA	20%	up to 5%	20-30%	5-10%
Sub-clone detection	1-3 out of 20 metaphases	1-5%	> 30%	> 30%	5~10% SV / 20% CNA	No	if more than 5%	20-30%	5-10%
SV detection									
Balanced translocation with fusion gene	☑	☑	No	No	☑	☑	☑	☑	☑
Unbalanced translocation with fusion gene	☑	☑	☑	No	☑	☑	☑	☑	☑
Balanced translocation with gene overexpression	☑	☑	No	No	☑	No	☑	☑	☑
Unbalanced translocation with gene overexpression	☑	☑	☑	No	☑	No	☑	☑	☑
Whole-arm translocation (Robertsonian type)	☑	☑	No	No	No	No	☑	No	No
Inversion (para- and pericentric)	☑	☑	No	No	☑	☑ if transcript	☑	☑	☑
Chromoanagenesis	possible	☑	☑ [1]	No	☑	No	☑	☑	☑
CNA detection									
CNA size	5-10 Mb	150 kb	30 kb	1 kb	500 kb	No	up to 35 kb in Hi-C	SNV	challenging
Haploidy, triploidy, tetraploidy,	☑	☑	aSNP	☑	☑+/- [2]	No	☑+/- [3]	☑	☑+/- [4]
Nullosomy, monosomy, trisomy, tetrasomy, etc.	☑	☑	☑	☑	☑	No	☑	☑	☑
Intra-chromosomal CNA	☑	☑	☑	☑	☑	No	☑	☑	☑
Other abnormality detection									
SNV	No	No	No	No	No	No	☑	☑	☑
CN-LOH	No	No	aSNP	No	☑	No	☑	No	No
Routine implementation									
Cost	low	low	low	low	high	low	high	high	high
Turnaround time	2 d	4 h to 2 d	3 d	1-2 d	3-4 d	3 d	3-4 d	15-21 d	15-21 d
Bioinformatic software availability	NA	NA	commercially available	commercially available	included with hardware	local development	local development	local development	local development

aCGH: array-based comparative genomic hybridization, aSNP: array-based single nucleotide polymorphisms, CBA: chromosome banding analysis, CMA: chromosomal microarray, CNA: copy number abnormality, CN-LOH: copy-neutral loss of heterozygosity, d: day, FFPE: formalin-fixed paraffin-embedded, FFPE-TL: FFPE-targeted locus capture, FISH: fluorescent in situ hybridization, Hi-C: high throughput chromosome conformation capture, LD-RTPCR: ligation dependent RT-PCR, LR-WGS: long read whole genome sequencing, MLPA: multiplex ligation-dependent probe amplification, OGM: optical genome mapping, SNV: single nucleotide variant, SV: structural rearrangements, UHMW: ultra-high molecular weight, RNA-seq: RNA sequencing, NA: not applicable.

[1] Chromoanagenesis may be suspected on highly variable CNA profile. [2] Theoretically not detectable but indirect detection possible for haploidies via the loss of heterozygosity detection tool. [3] Indirect detection possible by bioinformatic tool studying allele frequencies of inherited variants. [4] Depending on the bioinformatics tools used.

by grouping those that detect CNAs only, SVs only, or both (Fig. 1). The rapid advancement of these methods, which provide better CAs detection and a precise identification of abnormal cells chromosome patterns, has also led to an increased ability to detect catastrophic chromosome rearrangements with greater frequency. As the impact of these abnormalities on patient outcomes becomes more widely studied and is mentioned in other parts of these recommendations, we think that it is necessary to explain their mechanisms, which are naturally complex and may be difficult to grasp.

Alternative technologies to detect CNA

Chromosomal microarray analysis (CMA): comparative genomic hybridization (aCGH) and single nucleotide polymorphisms (aSNP) arrays

History

Chromosomal analysis on DNA chips emerged in the 1990s with the development of several chip preparation techniques for aCGH, resulting in the industrialization of three methods by Affymetrix (Thermofisher) [7], Agilent [8] and Illumina [9,10]. Later, aSNP was developed for SNPs detection using various methods: allele discrimination by hybridization, Illumina's "Golden Gate Assay", arrayed primer extension (APEX) or Illumina's Infinium assay.

Technical principles, advantages and limitations

CMA provides a high-resolution genome-wide analysis, especially when combining CGH and SNP probes, with robust and easily automated techniques for testing simultaneously a large number of patients within the usual timeframe of medical care.

Tested DNA can be extracted from various sources, including pretreated samples such as formalin-fixed paraffin-embedded (FFPE) tissue or cytogenetic pellet since CMA does not require cell culture and can be used in case of culture failure. Its sensitivity requires a minimum of 20–30 % of cells with abnormalities in bulk samples. Analyzing sorted tumor cell populations can counteract in part this limitation. However, it does not allow evaluation at the single-cell level, and lacks subclone detection and clonal architecture establishment.

Depending on the type of chip, the DNA is either tested alone (aSNP and aCGH) or "counterstained" with a diploid control DNA (aCGH only). Fluorescence intensity, proportional to the number of DNA copies, is measured for each genomic position tested by a scanner and analyzed by bioinformatics tools.

Two main applications are distinguished: genotyping and CNA detection. Allele discrimination and therefore the detection of uniparental disomy (UPD) or acquired copy-neutral loss of heterozygosity (CN-LOH) require aSNP analysis while CNA can be obtained using both aCGH and aSNP. Note that the information is only available for the covered regions and that the size of detectable CNAs depends on chip design and probe enrichment in the regions of interest. Particular attention must be paid to hypodiploidies or large hyperploidies which can be misinterpreted due to either technical or informatic issues, a common limitation to all DNA-based CNA detection techniques. Finally, CMA does not detect balanced structural abnormalities (Fig. 2).

Application in hematology

CMA has demonstrated significant diagnostic relevance in the context of acute lymphoblastic leukemia (ALL), myelodysplastic neoplasms (MDS) and myeloproliferative neoplasms (MPN) with the identification of classifying CNAs or passenger abnormalities which could have a prognostic impact, as summarized by Schoumans et *al.* [11]. In ALL, particularly B-lineage ALL, CMA is of paramount importance due to the high frequency of unbalanced abnormalities, a relatively frequent karyotype failure and substantial tumor infiltration. CMA achieves near 100 % detection of abnormalities [12] and provides more comprehensive information compared to CBA and FISH for the presence of submicroscopic deletion of the *IKZF1* gene and masked hypodiploidy [13]. It is noteworthy that a combination of CNAs involving 8 specific genomic regions (*EBF1, IKZF1, CDKN2A/2B, PAX5, ETV6, BTG1, RB1*, PAR1) has been shown to have prognostic value for the B-ALL stratification in the UKALL2003 clinical trial [14]. In acute myeloid leukemia (AML), the detection of CNAs and/or CN-LOH is estimated between 32 % to 68 % in normal karyotypes and may contribute to improved

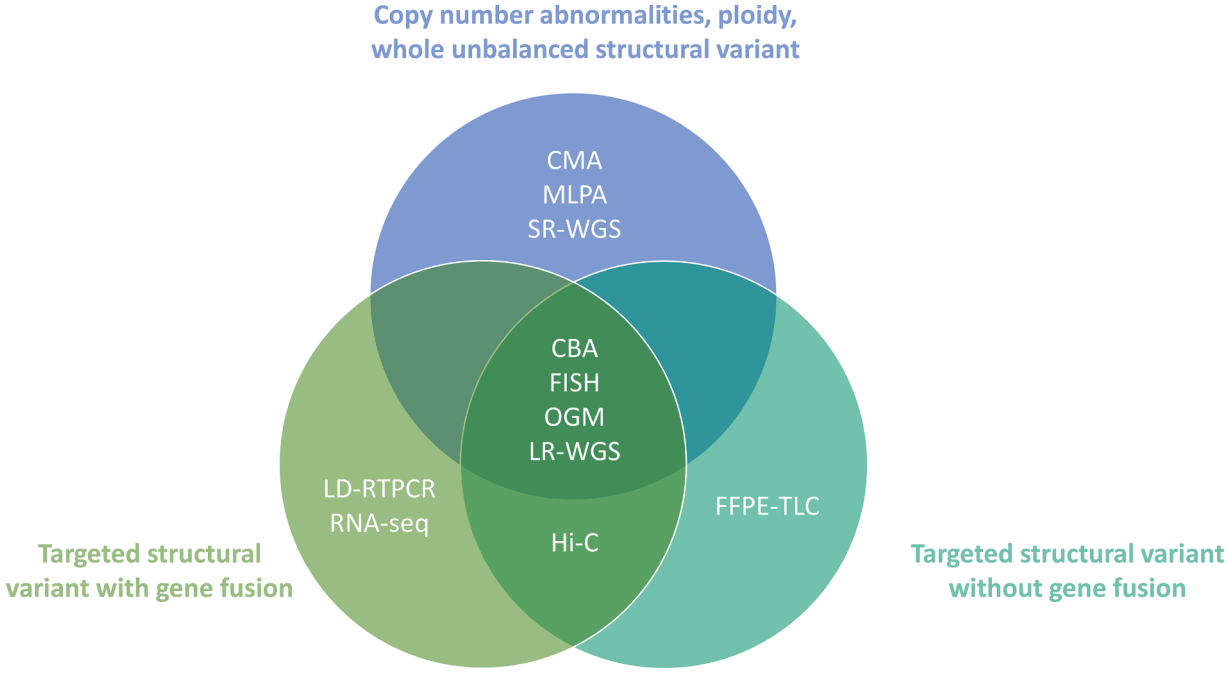

Fig. 1. Chart illustrating the detection capabilities of technology by type of chromosomal abnormality
CMA: chromosomal microarray analysis, MLPA: multiplex ligation-dependent probe amplification, SR-WGS: short-read whole genome sequencing, CBA: chromosome banding analysis, FISH: fluorescent in situ hybridization, OGM: optical genome mapping, LR-WGS: long-read whole genome sequencing, LD-RTPCR: ligation dependent RT-PCR, Hi-C: high throughput chromosome conformation capture, FFPE-TLC: formalin-fixed paraffin-embedded-targeted locus capture.

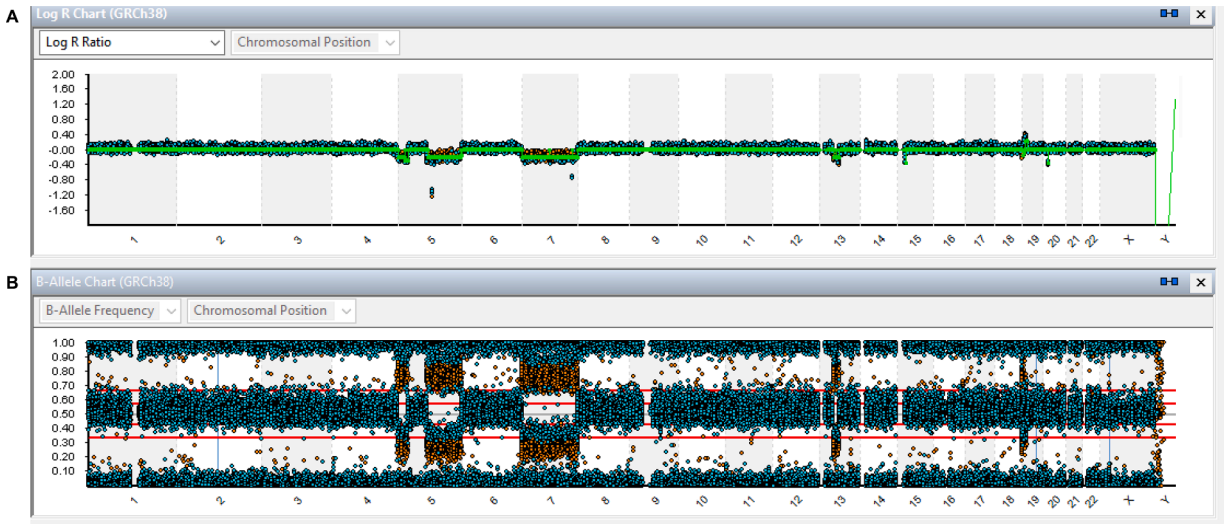

Fig. 2. Example of aSNP (Infinium CytoSNP-850 K, Illumina) in myelodysplasia in a female patient in the case of a failed bone marrow karyotype. a : Log R whole genome shows a deletion 5p15.3p13.3, a deletion 5q14.2q35.3, a monosomy 7, a deletion 13q14.11q21.1, a complex rearrangement of the short arm of chromosome 19. b : B Allele Frequency indicates the proportion of abnormal cells, 80 % for all abnormalities in this case. aSNP profile classifies this myelodysplasia as having a very poor prognosis according to R-IPSS.

prognostic stratification within the highly heterogeneous ELN intermediate-risk group (reviewed by Xu et *al.* [15]). The prognostic impact of CN-LOH is closely associated with the presence of recurrent mutations within the affected region. For MDS and MPN, the detection of abnormalities in cases with a normal karyotype is estimated between 33 % to 62 % with recurrent CNAs and CN-LOH regions, as reviewed by Kanagal-Shamanna et *al.* [16]. In MDS, it is either the detection of one or more abnormalities [17,18] or a cumulative size of the abnormalities > 100 Mbp [19] that is associated with poor prognostic value. In chronic lymphocytic leukemia (CLL), the interest of CMA is to reveal the genomic complexity and suspect chromothripsis event which characterizes aggressive diseases [20]. CNAs play an important role in the transformation of indolent lymphomas into high-grade lymphomas. However, current classifications and prognostic scores also include the detection of balanced translocations (*BCL2, BCL6, MYC, CCND1*, IG genes, etc.) or *TP53* subclonal deletion requiring the use of appropriate techniques [21].

Future and perspectives

Sufficient body of literature is available regarding the use of CMA in hematological malignancies to provide a comprehensive understanding of its main indications (ALL, MDS, MPN, CLL). Nonetheless, it is important to acknowledge the limitations of this technique, which include its inability to detect balanced SVs and subclonal abnormalities. It is advisable to complement this analysis with other techniques capable of addressing these shortcomings, particularly for ALL, AML and non-Hodgkin lymphomas. To promote consistency in clinical practice, the GFCH strongly recommends adhering to published technical recommendations and laboratory reporting guidelines [11,22], as well as using the current International System for Human Cytogenomic Nomenclature (ISCN) to describe relevant abnormalities.

Multiplex ligation-dependent probe amplification (MLPA)

History and technical principles

First described in 2002 [23], MLPA involves the hybridization of multiple oligonucleotide probe pairs to a DNA target, followed by ligation of the adjacent probe pairs only if they are correctly annealed to the target sequence. Subsequent PCR amplification of the ligated probes is performed, allowing their detection and quantification by electrophoresis or by next-generation sequencing (digital MLPA). This technique provides a valuable tool for analyzing hot-spot single nucleotide variants (SNVs), methylation status assessment (MS-MLPA), and to a cytogenetic extent, exon-level CNA.

Advantages and limitations

MLPA is able to simultaneously interrogate numerous targeted sequences in a single reaction, making it time-efficient and cost-effective. Furthermore, it provides a high level of specificity, even when applied to limited DNA quantities. However, the technique depends on the design of a limited number of specific probe sets unable to provide a genome-wide analysis. Similarly to CMA, its sensitivity is low (minimum of 30 % of tumor cells), and a low tumor sample infiltration or subclones can lead to an increase of false-negative results [24,25]. Additionally, MLPA is not be suitable for the detection of balanced SVs and genomic localization of duplications.

Application in hematology

MLPA probe sets have been developed for several hematological disorders and predispositions to such diseases. It is particularly valuable in B-ALL to quickly obtain CNAs information for the 8 genomic regions with demonstrated prognostic significance (refer to CMA section). A specific probe set for T-ALL with not only prognostic but also diagnostic importance is also available [26]. MLPA and digital MLPA have been tested in multiple myeloma (MM) and CLL with a high concordance and enhanced informativeness for the detection of CNAs when compared to FISH, thanks to the investigation of a greater number of targets in a single test. However, FISH remains superior to MLPA in detecting biallelic deletion in low tumor burden CLL [27–29].

Future and perspectives

MLPA is a quick and easy to perform technique for its implementation in a diagnostic laboratory. However, suffering from the same disadvantages as CMA, it is necessary to supplement MLPA with other techniques ensuring the detection of balanced SVs and subclonal abnormalities when they are involved in prognostic classifications.

Alternative technologies to detect SV

Chromatin conformation analysis

History and technical principles

Dekker introduced chromosome conformation capture (3C) in 2002, analyzing contact frequencies between genomic regions. This method fixes chromatin sites, performs digestion and proximity ligation, yielding 'chimeric' DNA containing 3D genomic information. Thus, DNA fragments that are distant in a linear perspective but colocalized in a spatial environment can be brought into proximity by ligation.

With NGS (next generation sequencing) implementation, high throughput 3C (Hi-C), a genome-wide approach, was developed [30].

Recently, a method similar to Hi-C has been published [31,32]: the FFPE-targeted locus capture (FFPE-TLC) with the advantage of studying SV and CNA on FFPE samples.

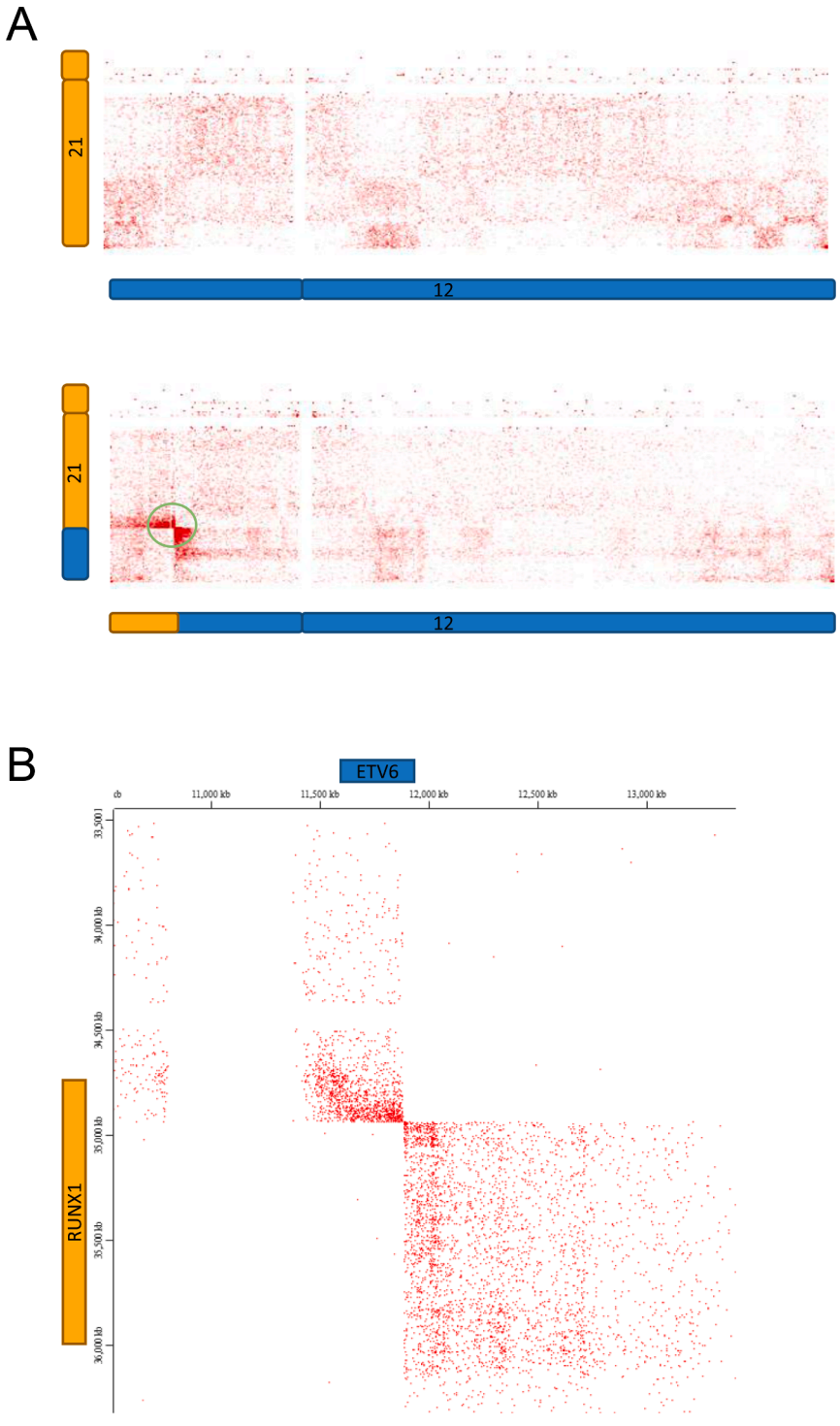

Fig. 3. Heatmap of normalized interchromosomal interaction frequencies between chromosomes 12 (blue) and 21 (orange) with Juicebox for visualization of high throughput chromosome conformation capture (Hi-C) data

A) Chromosome level : Up : Normal profile. Down : "Butterfly" appearance (green circle) showing an increase in contact frequencies due to a t(12;21)(p13;q22) *ETV6::RUNX1 B*) Gene level in hg19 coordonates: Butterfly showing involvement of *ETV6* and *RUNX1*.

Advantages and limitations

Hi-C show structural rearrangement on heatmaps. On those, SVs, like translocations or inversions, are seen as increased contact between distant genome regions. However, current detection relies on visual inspection, limiting its scope to predefined regions (Fig. 3) [33].

To note, recent bioinformatics tools automate detection, particularly proficient in identifying interchromosomal alterations, exhibiting strong correlations with FISH and WGS methodologies [34–37].

Concerning FFPE-TLC, several studies have confirmed its ability to identify SVs, with high performance compared to FISH or RNA-Seq (concordance 90 to 100 %). Moreover, FFPE-TLC successfully detected gene fusions that conventional methods missed due to limitations in detection or low sample quality [32,38].

Applications in hematology

Several studies have examined Hi-C in hematological malignancies, primarily as proof-of-concept rather than diagnostic validation. For example, Mallard et *al.* demonstrated t(12;21)(p13;q22) *ETV6::RUNX1* on heatmaps in four B-cell ALL patients using low-coverage Hi-C [39].

Other studies validated structural rearrangements in cell lines representative of various hematological malignancies [40,41].

The FFPE-TLC method was evaluated in 129 lymphomas, for six different loci (*BCL2, BCL6, MYC*, IGH, IGK and IGL), comparing its performance to both FISH and NGS [31]. It successfully identified expected alterations in all samples, exhibiting higher sensitivity (5 %) than the typical FISH rate (10 %). Moreover, FFPE-TLC outperformed NGS capture, the latter displaying a 25 % false negative rate.

Future and perspectives

Hi-C is currently difficult to implement in diagnostic routine due to its high cost, requiring significant sequencing capacity, and the lack of consensus analysis tools. Additionally, bioinformatics tools are often unable to detect small structural variants, typically less than 1 Mbp.

However, chromatin conformation analysis by FFPE-TLC technique could be an alternative approach to *MYC, BCL2, BCL6* and/or IG genes FISH in large B-cell lymphomas, especially in the absence of fresh tissue samples. Indeed, for FFPE samples, where DNA degradation and fragmentation hinder alternate methodologies such as LR-WGS or OGM, the time-consuming FISH technique remains the primary option. Therefore, FFPE-TLC seems promising for automated identification of SVs.

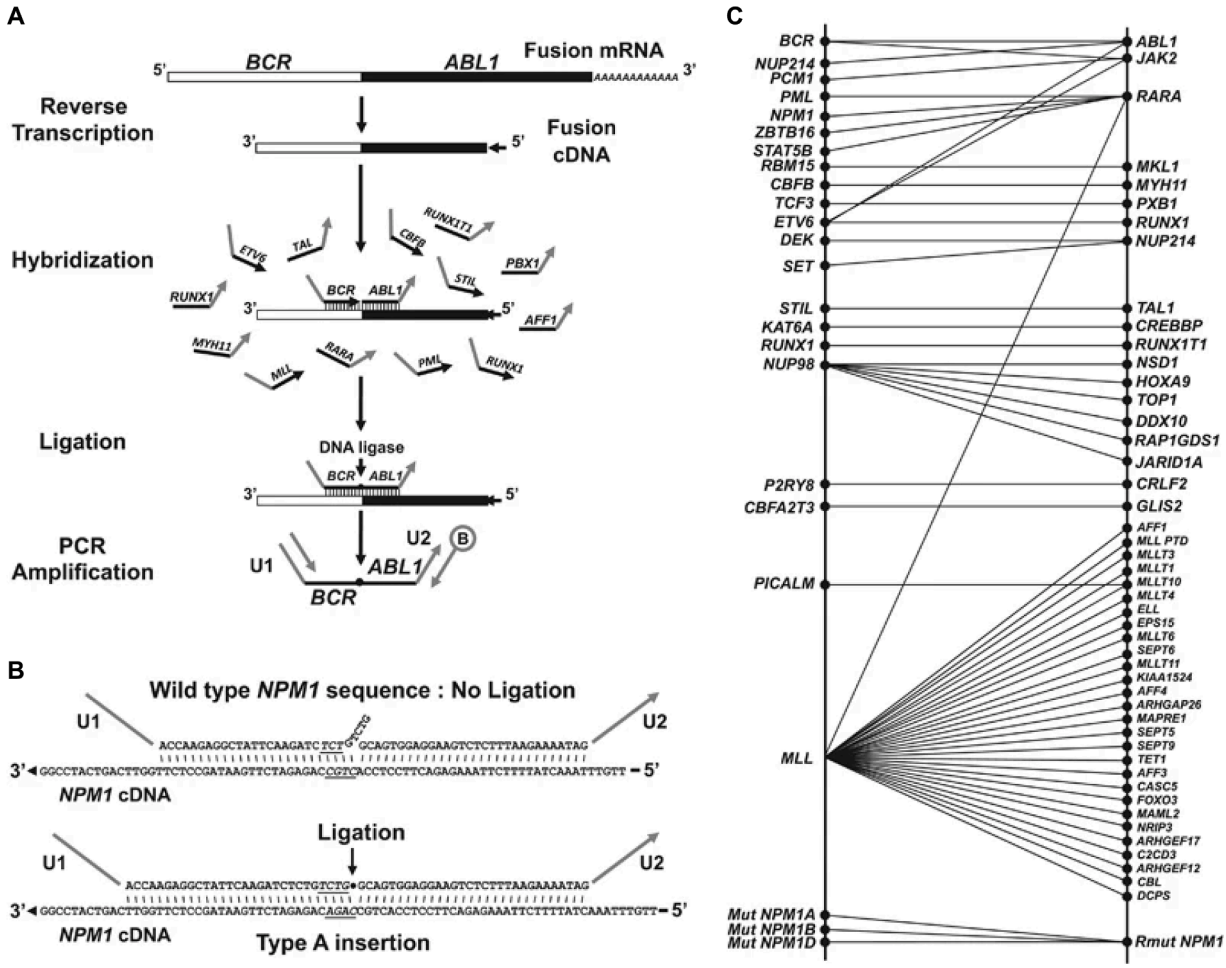

Fig. 4. Principle and design of the LD-RTPCR assay with courtesy of Philippe Ruminy [42]. (**a**) For the detection of fusion transcripts, total RNA samples are first converted into cDNA using a standard reverse transcription procedure. They are next incubated with oligonucleotide probes that correspond to the ends of the exons fused on hybrid mRNAs. If one fusion transcript is present, two probes hybridise side-to-side at the aberrant cDNA junction. A DNA ligase is next added to the reaction mixture to create a covalent link between these probes, which allows their amplification by PCR using primers that correspond to their additional tails. The two partners are next identified using pyrosequencing analysis. (**b**) For the detection of NPM1 mutations, left oligonucleotide probes harbouring four additional base pairs corresponding to type A, B and D insertions were designed, which can only be ligated to the adjacent right probes when these mutations are present. (**c**) Schematic representation of the assay. LD-PCR probes were initially designed for 66 different genes and three *NMP1* mutations. For most genes, multiple probes were designed on different exons to target different transcripts that result from the distribution of the genomic breakpoints within different introns.

LD-RTPCR: ligation dependent RT-PCR (Multiplexed targeted sequencing of recurrent fusion genes in acute leukemia)

The LD-RTPCR identifies gene fusions transcripts involving genes known to be implicated in structural alterations and whose breakpoints are known [42]. Its interest is to highlight classical fusions, rare or cryptic fusions that are not detected by CBA and FISH. This fast (three days) and inexpensive technique enriches the description of abnormalities in malignant hematologic diseases, but is not applicable in the case of deregulation of expression of a partner gene by juxtaposition of regulatory sequences of IG or TCR *loci* because this technique is based on transcript detection.

Technical principles

LD-RTPCR is a rapid and inexpensive ligation-dependent RT-PCR amplification assay that can detect multiple gene rearrangements. The technical principles are shown in Fig. 4.

In the case of fusion genes, the results provide information on the two partner genes identity, and on the breakpoints, which guides the selection of an appropriate assay for residual disease assay.

This assay is highly dependent on the knowledge of the exact junction in the fusion mRNA, as any variation at this site (as observed in *PML::RARA* fusion, where the breakpoint occurred within an exon) will prevent the detection of the rearrangement. However, since the vast majority of breakpoints occur within introns, most rearrangements can be detected by this method.

This method remains targeted and is not an exhaustive method applicable to all partners, although it may be updated for newly identified rearrangements in the future.

Future and perspectives

LD-RTPCR avoids the use of a large number of FISH probes that target translocations forming fusion genes, which are particularly important to look for in acute leukemia diagnosis.

RNA sequencing

In recent years, the application of NGS technologies notably whole transcriptome sequencing (commonly referred to as RNA sequencing or RNA-seq) has redefined the molecular profiles of hematological diseases. RNA-seq enables the identification of fusion transcripts, cryptic or not, sequence mutations and gene expression profiles in a single assay. RNA-seq can study the whole transcriptome or target a panel of anomalies relevant to specific pathologies. Of note RNA-seq is not optimal for assessing CNA [43–46].

It is important to note that chromosomal translocations involving non transcribed regions such as *IGH* promoter or enhancer elements are difficult to detect with RNA-seq [43].

Technical principles

Essentially, RNA-seq is based on RNA extraction. The following steps (amplification, ribosomal RNA depletion, library preparation, library sequencing) depend on the technologies used. The interpretation steps require bioinformatics strategy and biostatistical tools that allow, after the application of adapted filters, alignments and assemblies of reads reproducing transcriptome.

Advances in high-throughput sequencing techniques have led to the emergence of single-cell RNA-seq (scRNA-seq) methods, with the advantage of revealing the diversity of transcriptome profiles between cells [47,48].

Future and perspectives

RNA-seq is a powerful tool that can identify structural alterations and sequence mutations, with the possibility of establishing a better prognostic profile than conventional techniques and also therapeutic indications in a single technique. However, the equipment is expensive for a routine laboratory, and the time required to deliver results may not correspond to real-life treatment requirements. RNA-seq is best conceived as part of a genetic platform with a high throughput of tests.

Alternative technologies to detect both CNA and SV

Optical genome mapping (OGM)

History and technical principles

OGM was first described in 1993 by Schwartz et al. [49], who reconstructed the genome of the yeast *Saccharomyces cerevisiae* from DNA fragments obtained using the restriction sites of an enzyme. This technique was modernized in the 2010s and first applied to plant biology [50,51], then to human biology [52].

The current technology consists of the fluorescent labeling of ultra-high molecular weight (UHMW) DNA (from 150 kbp to 2.5 Mbp) by an enzyme based on a specific sequence, repeated about 15 times per 100 kbp in a pattern specific to each chromosomal region. The labeled DNA molecules are loaded onto a microfluidic chip with a network of nanochannels that allows the DNA to be linearized and the fluorescence to be read by a scanner. Once digitized, the images obtained are processed according to different pipelines in order to reconstruct the genome at the scale of an entire chromosomal arm. Two complementary algorithms are used for data analysis. The copy number variant algorithm for ploidy analysis is based on the molecules density in each region. The structural variant algorithm for qualitative genomic analysis is based on the position of fluorescent markers and their possible variations compared to the reference genome (Fig. 5).

Advantages and limitations

The DNA used for OGM must be of UHMW and therefore extracted using appropriate techniques. It is recommended either to extract UHMW DNA from a fresh sample within 24 h of sampling or to store the sample at −80 °C until extraction. UHMW DNA can be extracted from frozen biopsies. OGM can detect structural abnormalities, balanced or unbalanced, intrachromosomal or interchromosomal, with a theoretical minimum resolution of 500 bp to 5 kbp, depending on the pipeline used for genome assembly. Recent technology updates allow for detection of UPD and CN-LOH. OGM can also detect CNAs with a theoretical minimum resolution of 500 kbp. For the analysis of mosaic anomalies, the maximum depth achieved in a single run is approximately 300 to 400x. Like CBA, OGM is a genome-wide technique, but without the need for prior cell culture. Technical limitations are mainly related to the highly repetitive nature of the genome, starting with telomeres and centromeres, which theoretically make it impossible to detect translocations of entire chromosomal arms as roberstonian translocations. Random difficulties in identifying abnormalities in the PAR region have also been described [53,54]. Cases of ploidy abnormalities (hypodiploid clone, tetraploid, triploid etc.) are often poorly analyzed computationally by the algorithms. Finally, detecting large CNAs above 1 Mbp with low mosaicism levels presents a significant challenge [53,55,56].

Implementation in the routine laboratory

The hands-on time is a minimum of two days up to four. Of note, UHMW DNA extraction robots are available. The migration and image acquisition time in the scanner is less than one day for a desired depth of 400X with up to three samples in parallel. Bioinformatics data processing takes another half-day. Currently, the throughput is estimated at six to nine samples per week and per scanner, still much lower than that of conventional cytogenetics. Throughput improvements and optimizations are under development. In terms of cost, OGM is approximately twice as expensive as CBA and equivalent to CBA with two to three FISH analyses. Considering the potential of this technology and the multiplication of FISH in numerous pathologies for exhaustive stratification (pediatric ALL, multiple myeloma ...) this represents a contained cost.

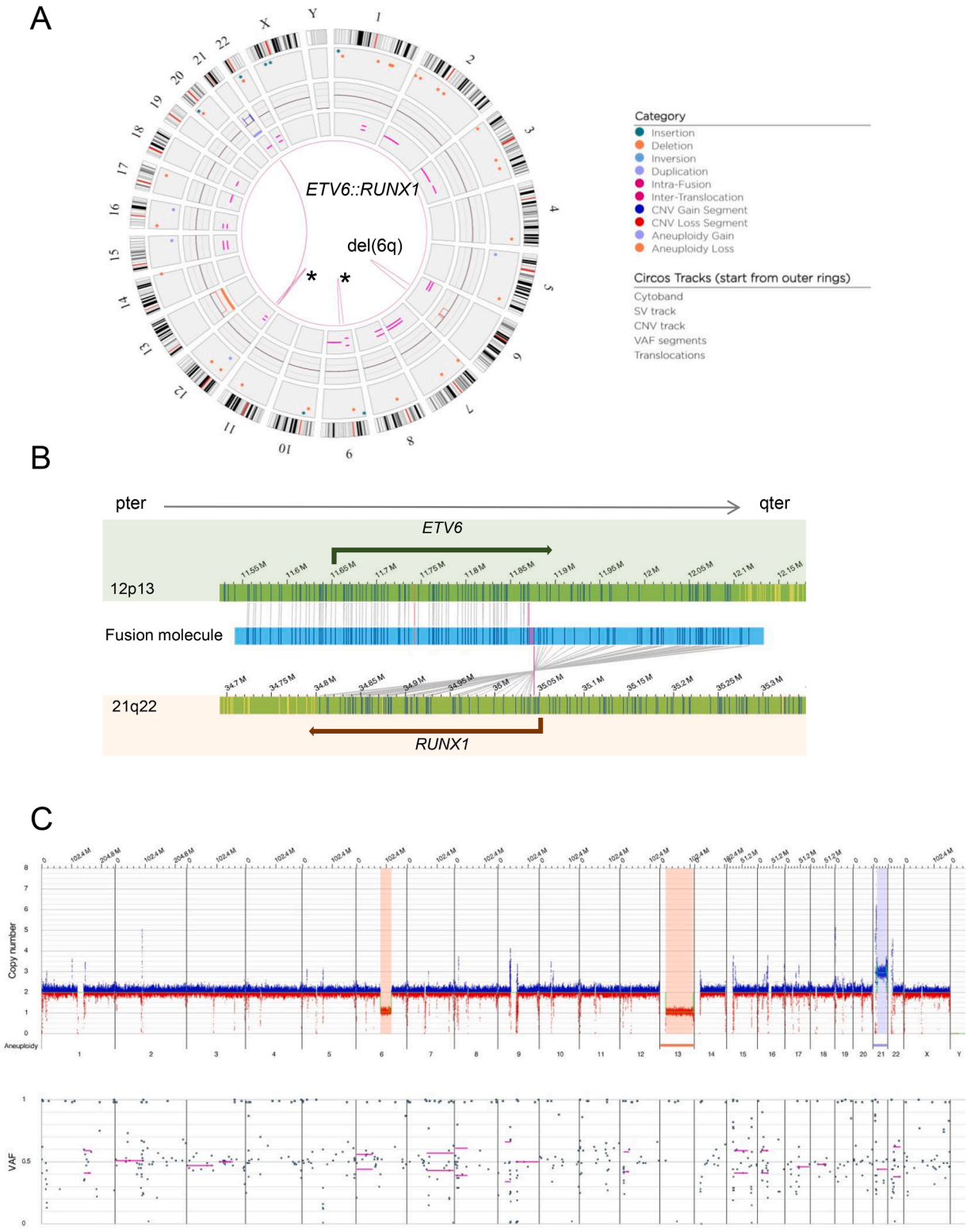

Fig. 5. Optical genome mapping results of a B-ALL t(12;21)(p13;q22) *ETV6::RUNX1* patient. A) Circos plot representation and its specific legend B) *ETV6::RUNX1* fusion molecule C) Whole genome copy number view and VAF calculation showing 6q deletion, monosomy 13 (red background) and trisomy 21 (blue background). * false positive intrafusion.

Applications in hematology

In just a few days, this technology makes it possible to obtain, in a single study, data at least equivalent to that of aCGH/aSNP, transcript search, FISH and CBA, within the aforementioned limits. Several studies have demonstrated the ability to combine these analyses, mainly in ALL [53–55], AML and MDS [56–60]. In ALL, of the 63 cases analyzed in the three studies comparing OGM with standard techniques (CBA, FISH, CMA and transcript search by LD-RTPCR or RNA-seq), OGM detects more than 90 % of the abnormalities described by standard techniques and provides additional information for a better prognostic stratification

in some patients. This includes Ph (Philadelphia, *BCR::ABL1*)-like abnormalities and other gene fusions with a poor prognosis, making OGM a practical substitute for FISH analysis recommended in current pediatric B-ALL treatment protocols. However, in all three studies, there were difficulties in detecting *CRLF2* (Xp22.3/Yp11.3) rearrangements and in identifying or correctly classifying ploidy abnormalities. In AML, four studies compared OGM with standard techniques (CBA, FISH and CMA) in 184 cases, also showing excellent concordance. OGM found between 85 % and 95 % of the abnormalities detected by the standard techniques. These data clarified the diagnosis and/or the prognosis of patients with, for example, *NUP98* (11p15) and *MECOM* (3q26) rearrangements, *KMT2A*-PTD (partial tandem duplication) abnormalities and a *RUNX1::RUNX1T1* fusion masked by an insertional mechanism. The main limitation was the detection of aneuploidies present in a low number of mitoses on CBA. Similarly, the analysis of 136 cases of MDS in three studies highlighted the very good performance of OGM in these conditions. In the series of 101 cases by Yang et *al.*, OGM provided additional information compared to CBA in 34 % of patients. In 17 % of patients, this additional information changed the Revised International Prognostic Scoring System [61]. OGM is also helpful in cases where cytogenetics present difficult interpretation, as seen in chromoanagenesis, and can provide supplementary data, as in CLL [62] or myeloma [63]. This technology is also valuable in pathologies with low cell proliferation since it removes the necessity for cell culture. Specifically, OGM appears to be a promising tool for identifying tyrosine kinase gene fusions in MPN [64,65].

Future and perspectives

In view of the data available in the literature, the GFCH would like to suggest the use of OGM as a first-line method for the diagnosis of acute leukemias (ALL and AML) and MDS, as a complement to CBA and as an alternative to FISH analysis recommended in these conditions, for laboratories wishing to implement it.

For other indications, additional data are needed. However, OGM can be used in addition to CBA to specify complex SVs and resolve complex karyotypes (CK). It could also replace FISH or CMA in cases where it is necessary to identify a large number of potential SVs leading to fusion genes or expression deregulation, or small CNAs. A first OGM nomenclature has been published and should be used [66].

In the near future, OGM may become a first-line cytogenetic tool for many hematologic malignancies. OGM represents a significant advance in describing CAs, and can be regarded as a next generation cytogenomic (NGC) analysis in the same way that NGS has transformed sequencing methodology.

Whole genome sequencing applied to onco-hematological cytogenetics

We will primarily focus on WGS approaches, which are better suited to detect CAs. From the cytogeneticist's point of view, we focus only on the ability of WGS to detect CNAs and SVs. Of course, the different WGS technologies can also detect SNVs, which are necessary for various diagnostic and prognostic classifications and are of therapeutic interest. Some technologies, such as Oxford Nanopore technology (ONT), additionally analyze methylation, which is of increasing interest in hematological malignancies. Every molecular data provided by WGS is thus complementary to cytogenetic data.

Short-read whole genome sequencing (SR-WGS)

The effectiveness of the short-read approach based on the sequencing of short fragments, is highly dependent on the choice of library preparation and bioinformatics tools for variant calling. In 2021, a study evaluated SR-WGS (60X) for prognostic stratification of 235 patients with myeloid pathology (AML and MDS) compared to cytogenetic analysis [67]. Compared to CBA, this strategy showed 100 % concordance for the detection of recurrent translocations and CNAs and identified additional SVs (6 % for translocations and 10 % for CNAs) which changed/redefined the prognostic group in 15 % of cases.

However, a limitation of this study was the resolution level of CNAs set at 5 Mb. This low level of resolution can be explained by the difficulty in detecting small CNAs from SR-WGS data for several reasons: variable genome coverage; alignment bias for deletions; limitations in aligning repeated regions. Interestingly, Haferlach *et al.* described that sensitivity increased with higher sequencing depth. They studied 440 samples at 100X coverage and were able to detect 96.6 % of the CAs considered. The CAs that were not detected had a VAF of less than 35 % [68]. However, there is ongoing debate regarding the clinical significance of VAF and coverage thresholds. It is therefore understandable that the evaluation of bioinformatics tools is as important as the type of library chosen.

Long-read whole genome sequencing (LR-WGS)

LR-WGS is based on two main technologies: PacBio's technology, which is based on fluorescence detection during the incorporation of labeled nucleotides by a polymerase (fixed on a support), and ONT, which uses electrical signaling to modify a biological pore by passing a DNA strand according to its base content [69,70]. Compared to SR-WGS, it reduces multiple alignments and can study repetitive sequences and complex SVs. It has also been shown that LR-WGS is more accurate in identifying SVs than traditional SR-WGS [71].

In the field of onco-hematology, this technology has been employed to detect translocations and to describe breakpoints by sequencing fragments exceeding 20 kb, facilitating identification of relevant genes involved [72]. It also enables rapid analysis of a fusion gene in AML [73]. The main limitation is the depth of sequencing obtained (generally around 10X), not well adapted to a somatic approach. Therefore, a low coverage approach (8X) was evaluated on two AMLs using ONT, but was not sufficient to detect all SVs [74].

Implementation in the routine laboratory

WGS implementation, by SR-WGS or LR-WGS approach, requires a multidisciplinary team with strong bioinformatics skills to fully exploit the possibilities of CA detection. Furthermore, CNA and SV analyses require a cytogenetic approach by personnel experienced in detecting these types of abnormalities. ONT has the advantage of being more accessible and easier to implement, in the absence of ready-to-use bioinformatics solutions that are almost non-existent today. In addition, the cost of LR-WGS remains high compared to the combination of CBA and FISH, for an information gain that remains modest in most cases. It should be noted that the use of these technologies requires good quality, non-fragmented DNA. Extraction can be performed on tissue biopsy and circulating tumor DNA (ctDNA) [75]. However, LR-WGS interest in ctDNA is limited by DNA fragmentation.

Future and perspectives

LR-WGS based on ONT can be an alternative for identifying SVs in genes of interest, as well as evaluating CNAs in the absence of a CMA/OGM platform. Combining SR-WGS and LR-WGS techniques appears to be a valuable approach to achieving a comprehensive cytogenetic analysis [76].

Chromoanagenesis: chromothripsis, chromoanasynthesis and chromoplexy - improved detection using alternative technologies

The emergence and utilization of more resolutive genome-wide technologies have unveiled novel categories of massive and complex chromosomal and genomic alterations. Some of them are characterized by the simultaneous occurrence of multiple structural rearrangements affecting one chromosome or chromosome segments during a single, or few, catastrophic events. Referred to collectively as 'chromoanagenesis' (signifying chromosome rebirth), this novel class of chromosomal alterations encompasses three distinct phenomena: chromothripsis,

chromoanasynthesis, and chromoplexy.

These three entities are distinct by their molecular aspects, specifically CNAs, associated SVs, and the DNA repair mechanisms involved. Differentiating chromoanagenesis from other classes of complex rearrangements is important because it is typically associated with a poor prognosis. Moreover, understanding the differences between the three mechanisms is essential for choosing the appropriate technological approach to identify the main characteristic features. We present essential elements for comprehension and characterization of these phenomena.

Chromothripsis

This mechanism is extensively studied and the best understood. It was first described in 2011 [77]. It is a mutational event induced by multiple double-strand breaks during a single catastrophic event between a few chromosomal segments followed by a random unordered reassembly of the DNA fragments to form a complex derivative chromosome, mostly associated with genomic losses. Precise sequence analysis of the joining sites indicates that DNA fragment reassembly is induced by recombination mechanisms such as classical non-homologous end joining (c-NHEJ) or alternative forms of end joining (alt-EJ). In 2013, Korbel proposed six criteria to help standardize the definition of chromothripsis within complex chromosomal rearrangements. To distinguish stepwise from one-off events, at least two criteria must be met [78]. The analysis of the etiology of chromothripsis has led to the identification of several cellular mechanisms capable of initiating these processes. The two main models are the micronucleus hypothesis and the telomere crisis one, which are ideal substrates for chromosome pulverization either by premature chromosome condensation (PCC) or by exonuclease action (Fig. 6). A third model, aborted apoptosis, has been proposed, but it is difficult to explain why chromothripsis is often limited to a restricted chromosomal region [79–82].

Chromoanasynthesis

Chromoanasynthesis is a distinct form of "one-step" chaotic chromosomal rearrangement first described by Liu in 2011 [83]. It is related to replication stress with replication fork dysfunction and involves multiple template switching events driven by microhomology-mediated break-induced replication (MMBIR) or fork stalling and template switching (FoSTeS) mechanisms. Both mechanisms are based on microhomology sequences that may represent a mechanistic signature at breakpoints. Together with the presence of duplicated or triplicated focal regions associated with lost and neutral segments in the rearranged chromosome, these are two major differences with chromothripsis [84].

As with chromothripsis, the micronuclei model provides an attractive explanation for the genesis of chromoanasynthesis. Indeed, delayed chromosome replication in micronuclei is asynchronous and defective compared to the primary nucleus, leading to PCC upon entry into mitosis and resulting in replicative stress. In fact, in this model, chromothripsis and chromoanasynthesis could not be mutually exclusive.

Chromoplexy

Chromoplexy is a multiple inter- and intra-chromosomal translocations and deletions in a generally multistep process. This mechanism can involve up to eight chromosomes, resulting in the formation of derivative chromosomes. In contrast to chromothripsis, deletions are less frequent and more scattered. Chromoplexy breakpoints cluster with regions of DNA transcription or replication and open chromatin configurations but lack microhomology, unlike chromoanasynthesis. Chromothripsis and chromoplexy processes can occur simultaneously or asynchronously in the same cell resulting in different chromosome complexity profiles. These mechanisms lead to deregulation of many genes involved in neoplasia and are associated with significant tumor aggressiveness.

All these phenomena have been described in many hematological diseases (first chromosome 4q abnormality in CLL). Chromothripsis is detectable in 20–30 % of newly diagnosed multiple myeloma (MM) using WGS and could represent an early driver event. It remains relatively stable over time and is emerging as one of the strongest features able to predict both the progression free survival and the overall survival [85,86]. In AML (6 %) worse prognosis is associated with CK, and the complex model of intrachromosomal amplification of chromosome 21 in B-ALL [87].

Despite the chromosomal cataclysm represented by chromoanagenesis, some cells survive having acquired new genotypic traits involved in tumorigenesis: deletion of tumor suppressor genes, amplification of oncogenes or emergence of fusion genes with oncogenic properties or neoantigens [88]. These characteristics may represent new therapeutic opportunities in the future, such as a targeted immune response against neoantigens or the exploitation of metabolic dysfunctions resulting from random rearrangements [89].

Technical considerations

The frequency of chromoanagenesis displays variability across studies that can be attributed to two factors: the diversity in techniques used and the inadequate selection criteria, often reliant solely on the CNAs count. While a high number of CNAs in a confined chromosomal region may be considered suspicious of chromoanagenesis, particularly chromothripsis and chromoanasynthesis, this metric alone is insufficient to define it. For example, early CMA studies, which focused on the detection of CNA as the definition of chromothripsis, largely underestimated these mechanisms due to the limited resolution inherent in these techniques and to their inability to highlight balanced rearrangements.

In fact, the detection of SVs and the analysis of the sequence of rearrangement breakpoints (with the identification of microhomologies) are essential for defining the underlying repair mechanisms—fundamental elements in the genesis of chromoanagenesis phenomena. The identification of chromoanagenesis relies not on a specific count of deletions that guarantees a diagnosis but rather on a compilation of evidence accessible through modern technologies.

The WGS approach appears to be the technology of choice for identifying chromoanagenesis phenomena, as it enables the detection of all types of rearrangements, whether balanced or not, and the study of breakpoints sequences. Thus, it has been shown that tumors with chromothripsis are depleted in microhomologous sequences compared to tumors without chromothripsis, which confirms the involvement of the NHEJ repair mechanism [90].

With this technological change, the prevalence of chromothripsis in hemopathies, especially in MM, has been revised upward from 2 % with aSNP to 24–33 % with WGS [90,91].

Although WGS may become the gold standard technology in the near future, there are other interesting approaches based on OGM [92] or Hi-C [93] to detect chromoanagenesis phenomena. In the future, a computational approach like OGM or Hi-C with LR-WGS holds promise and might prove to be beneficial [70].

Conclusion

The new ICC 2022 and WHO-HAEM5 classifications [1,2,4,5] provide a more precise classification of hematological malignancies by incorporating CAs. They also involve mutational profiles and cryptic SVs, which require complementary diagnostic tools to gather all the genetic information necessary for optimal patient management. Although CBA and FISH remain the gold standard in hematology, both in daily practice and in clinical protocols, several alternative cytogenetic technologies aim to overcome CBA and FISH shortcomings or even supersede them in the short to medium term. WGS approaches have the

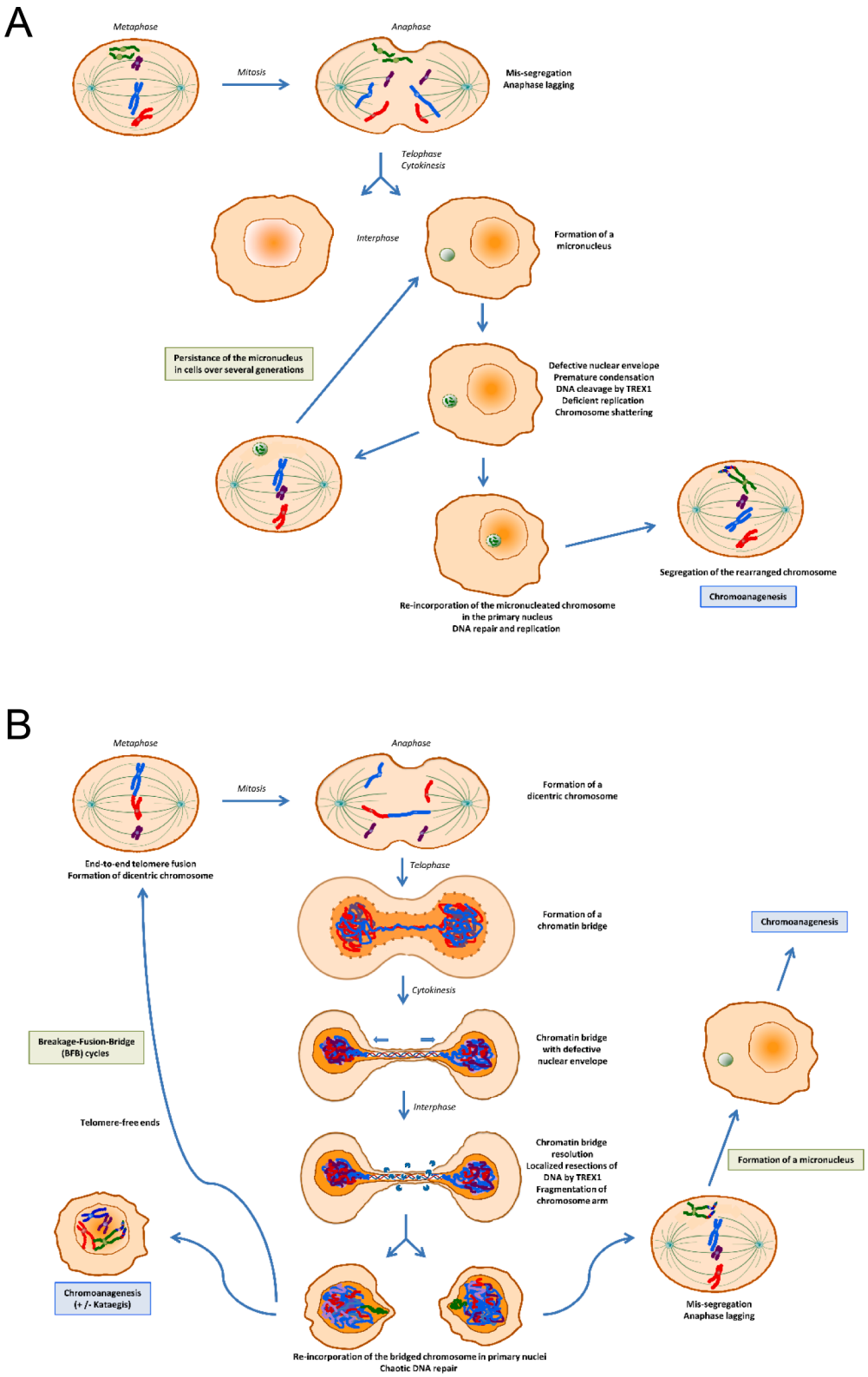

Fig. 6. Illustrations of cellular mechanisms leading to chromoanagenesis with courtesy of Franck Pellestor
A) Micronucleus model : a lagging chromosome is incorporated into a micronucleus. Through a combination of mechanisms such as abnormal replication and exposure to exonuclease, the sequestered chromosome is shattered, followed by a reassembly B) Telomere crisis starts with the formation of a dicentric chromosome that cannot be properly segregated at anaphase, resulting in the formation of a DNA bridge. Resolution of the DNA bridge is mediated by either an exonuclease, or by mechanical disruption leading to DNA fragmentation subject to repair mechanisms leading to chromoanagenesis.

potential to eventually supplant all techniques for visualizing CNA, SVs and SNVs, while techniques for analyzing epigenetic factors, such as aberrant methylation, should also be implemented. Currently, no single test can detect all genetic abnormalities, so laboratories must organize their techniques based on their capabilities and the number of patients under their care. This ensures satisfactory results within a reasonable timeframe for patient therapeutic management. The GFCH considers that cytogenetics remains a discipline and cannot be reduced to a simple set of tools. The cytogeneticist's analysis remains central to the interpretation of these tests. Furthermore, innovative technologies may enable the discovery of novel genetic abnormalities, which will require clinical evaluation through comprehensive studies to redefine prognostic classifications if necessary. Additionally, it is important to consider that alternative or innovative technologies may reveal germline variants of clinical significance. These findings should be interpreted in accordance with established international guidelines and local regulation. Ultimately, each laboratory must determine the optimal range of techniques that aligns with its own practices and daily activities in order to enhance patient care.

CRediT authorship contribution statement

Valentin Lestringant: Writing – original draft, Conceptualization. **Hélène Guermouche-Flament:** Writing – original draft, Conceptualization. **Mélanie Jimenez-Pocquet:** Writing – original draft, Conceptualization. **Jean-Baptiste Gaillard:** Writing – original draft, Conceptualization. **Dominique Penther:** Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare no Conflict of Interest.

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