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OBM Genetics

Review

Next-Generation Sequencing-Based Testing in Diagnostic Oncohematology: Untangling the Knots

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Academic Editor: Joep Geraedts

Special Issue: Genetic Testing

OBM Genetics
2019, volume 3, issue 3
doi:10.21926/obm.genet.1903088

Received: April 1, 2019
Accepted: July 30, 2019
Published: August 9, 2019

Abstract

With the advent of next-generation sequencing (NGS), genomic profiling of tumors has been gradually introduced into the clinical setting and has become a standard in cancer care. NGS allows convenient, rapid, and inexpensive sequencing and the commercially available NGS panels enable the detection of single or global genomic alterations of germline and somatic origin. Today, genomic mutation profiling using NGS is indispensable for disease evaluation and prediction of prognosis or responsiveness to cancer therapy.

However, the challenges encountered when applying NGS testing for diagnostic use are numerous, particularly the ones concerning interpretation and reporting. The current recommendations concern NGS mutation profiling in hereditary genetics and somatic genetics applicable to solid tumors; however, clear guidelines are lacking in regard to the specific challenges encountered in the application of NGS mutation analysis to hematological malignancies.

In order to bridge this gap, the present report proposes recommendations for handling the specific challenges encountered while applying NGS mutation testing for the confirmation of

diagnosis, risk stratification, and the prediction of response to therapy in the routine diagnostic setting for hematological neoplasia.

Keywords
NGS panels; somatic genetics; oncohematology; variant interpretation; recommendations; laboratory diagnostics

1. Introduction

The detection of acquired genomic mutations using next generation sequencing (NGS) has become increasingly important for disease evaluation and prediction of prognosis or responsiveness to cancer therapy [1, 2]. It has, therefore, been gradually introduced into the clinical setting, and has become indispensable in cancer care, even though only a limited number of mutations are considered actionable in the field of oncohematology [3, 4].

The high throughput capacity of NGS has overcome the main limitation of mutation screening using conventional molecular methodologies and allows easier, faster and cheaper sequencing [5]. A large selection of panels enabling the detection of single or global genomic alterations, of germline and somatic origin, have become commercially available in the past few years [6, 7]. NGS has the potential to allow tailoring oncological treatments, taking into account each patient's features and each cancer genomic alteration, and may eventually lead to large scale "precision medicine".

Furthermore, the introduction of NGS into clinical setting contributed to the discovery of an ever-growing list of genes predisposing to hematological neoplasia and an increase in the knowledge regarding the inherited genetic causes of hematological neoplasia. For instance, primary immunodeficiency disorders (PID) have been increasingly recognized to be associated with hematological malignancies, and therefore, increased awareness regarding the possibility of development of leukemia or lymphoma is warranted when providing care to the patients with PID [8]. Today, hereditary genetic analysis in hematological disorders is a rapidly emerging field, although, in the present report, the focus has been limited to the challenges encountered in regard to acquired mutations analysis in oncohematology.

The NGS technology enables simultaneous identification of different types of mutations, including single nucleotide variants (SNVs) as well as small insertions or deletions. The NGS technology has already demonstrated, in a research environment, its capability to identify all types of genomic aberrations clinically relevant for cancer patient management [9-11]. Nevertheless, analyses performed using conventional cytogenetics, fluorescence *in situ* hybridization (FISH), and/or microarrays continue to be the methods of choice for the detection of larger genomic rearrangements, such as copy number alterations (CNAs) and structural variants (SVs), in the majority of oncohematological diagnostic laboratories [12-14]. Nevertheless, NGS approaches can be used to screen a large number of recurrent fusion genes, simultaneously but reliably, and are currently implemented for clinical use. Somewhat more challenging is the implementation of the analyses for the identification of rare and complex rearrangements as well as genome-wide CNA and copy neutral loss of heterozygosity (CN-LOH); however, rapid whole-genome sequencing

(WGS) and/or full transcriptome analyses may become available at a reasonable price and on a wider scale in the near future, eventually enabling complete genomic profiling for each cancer patient [15-17].

Since targeted gene panels are the most widely used NGS panels for mutation screening in a routine diagnostic setting for hematological neoplasia, the discussion in the present report has been limited to this type of NGS analysis. Current recommendations concern NGS mutation profiling in hereditary genetics and somatic genetics applicable to solid tumors [18, 19], although clear guidelines are lacking in regard to the specific challenges encountered in the application of NGS mutation analysis for risk stratification in hematological malignancies. The aim of the present report is to highlight the specific difficulties encountered when establishing NGS in an oncohematological laboratory, and more importantly to propose recommendations regarding how to handle the specific challenges concerning the interpretation and reporting of acquired mutations in hematological neoplasia.

2. NGS Strategy

Several NGS technologies have been developed over the years. Among these, short-read sequencing technologies are mostly used in the clinical field, and Illumina and Ion Torrent are the main platforms used. Each NGS approach has its own advantages and disadvantages, and it is, therefore, important to clearly define the requirements of the laboratory in order to select the most suitable NGS strategy. When introducing NGS into a routine diagnostic setting, several points having the potential to influence the choice of NGS strategy should be considered, including the following:

- the expected volume of samples;
- the expected turnaround time;
- the genomic size/number of genes to be sequenced;
- the amount of DNA generally available in each sample;
- the expected depth of coverage;
- the requirement of flexibility for combining several panels in the same sequencing run;
- the cost per sample;
- the types of genomic variations which should be detected;
- the degree of technical and bioinformatics expertise in the laboratory.

Among all these points, the crucial ones to consider in the context of oncohematology are the depth of coverage (discussed in the coverage section) and the turnaround time. A rapid mutation profiling is important for patient management as it helps classifying the disease and predicting the outcome [20, 21]. Moreover, the presence or absence of targetable mutations may affect the therapeutic decision (e.g., *IDH1* and *IDH2* inhibitors in acute myeloid leukemia (AML) [22]). Therefore, it is important to have access to the status of mutation at the time of diagnosis, particularly for acute leukemia, within 10 to 14 days, or at least within the first treatment cycle [23].

Advantages and disadvantages of the two mainly used NGS technologies in regard to the points to consider for selecting the NGS strategy to be applied have been summarized in Table 1.

Table 1 Comparison between the two mostly used sequencing platforms for the implementation of NGS in a clinical setting in the context of oncohematology (adapted from [24, 25]).

	Illumina (Hybrid capture)	**Ion Torrent (Amplicon)**
Input DNA	As low as 50ng	As low as 10ng
Throughput	Very High	High
Library preparation time	Days	Hours
Sequencing time	1–2 days	Hours
Ultradeep sequencing	Not practical	Yes
Sample capacity	Very high	Flexible
Variation in coverage among targeted regions	Small	Large
Off target sequences	Medium	Very low
Accuracy	High	Moderate
Reported error rate	0.2%–0.5%	~1%
Hotspot SNV detection	Yes, but limited for subpopulations	Yes
Any SNV detection	Yes	Yes
Delins detection	Yes	May be suboptimal near the end of amplicons
CNV detection	Yes, but may be affected by GC content	Yes, but may be subject to amplification bias
SV detection	Yes	No

The current NGS technologies offer the possibility of sequencing the entire exome or even the whole genome. However, this may affect the sensitivity of the test, since the depth of coverage would become restricted. Furthermore, these techniques are expensive, and the huge amount of data produced requires an extended time for analysis, which is not always suitable for a clinical setting. The aforementioned reasons, therefore, restrict the application of whole-genome and exome sequencing to a research setting, for the identification of the most frequently altered genes in different cancer types, identification of driver genes, contributing to a better understanding of tumorigenesis, and for the development of potential therapeutic targets [26-28]. Despite the extensive research undertaken for characterizing the tumor genomes, e.g., The Cancer Genome Atlas (TCGA), it has been discovered that only a limited number of genes have an impact on patient management, since large cohorts of patient are required in clinical trials to predict the clinical outcome and bring improvement in the global treatment strategies [29, 30]. With regard to the treatment strategies, there are numerous solid tumors for which patients eligible for targeted

drugs can be identified on the basis of the detection of "actionable mutations". On the contrary, in the treatment of hematological neoplasia, mutation profiling is used for risk stratification, as only a few mutations are actionable (i.e., only a few mutations have a targeted therapy currently) [31, 32]. However, mutations in several recurrently mutated genes would impact prognosis and, therefore, guide the clinicians while making their therapeutic choices, which could lead to, for instance, bone marrow transplantation. Therefore, a targeted gene panel sequencing approach is currently sufficient to investigate the clinically relevant genes, and also enables inexpensive testing with a rapid service.

3. Quality Control

In somatic NGS mutation analyses, regardless of the gene panel content or the technology used, it is important to ensure reliable detection of low-frequency variants in order to detect subclones and monitor the treatment response. Therefore, quality variability has a much higher impact on the somatic variant analysis and interpretation in comparison to germline mutations analysis. Moreover, if the sequencing quality is not optimal, the risk that low-frequency variants remain undetected increases, and sequencing artifacts could become hardly distinguishable from the truly acquired variants.

Quality control is based on the initial platform validation and determination of its thresholds and limits. The step of quality control is, therefore, crucial for achieving reliable routine analyses. Quality controls could be defined at three levels: quality of the NGS run, quality of each sample, and quality of each retained variant. Several parameters may be defined for each of these three levels, depending on the sequencing platform and the bioinformatics pipeline or software used. It is strongly recommended to establish these parameters during the initial validation and to follow them during each run, for each sample, and for each retained variant. Stable quality parameters indicate reliable sequencing, while unfulfilled quality parameters indicate a technical problem and may lead to the detection of false positives or false negatives. It is important to establish quality criteria for each of the three levels, and identify when the screened samples do not reach the predefined quality criteria for diagnostic purposes, in order to undertake adequate interventions subsequently. Samples should be rerun in case of failure, or accepted, and interpreted with caution in the case when it is not possible to rerun a sample due to lack of material. The quality controls described in the present report are based on sequencing using the Ion Torrent platform, although these approaches are generally applicable to other platforms as well.

Quality Parameters for the NGS Run: quality parameters for the run are partially dependent on the platform, although these may include the loading of the chip/flow cell, read length (mean, median, and mode), percentage of aligned reads, mean accuracy, alignment quality, base quality, among others.

Quality Parameters for the Samples: sample quality parameters include the number and quality of the bases, number of reads, read length, number of mapped reads, percentage of reads on target, mean read depth, uniformity, among others.

Quality Parameters for the Variants: the variant quality parameters include coverage, strand bias, base call quality (Phred scores), identification of error-prone regions (GC repeats, homopolymers, end of reads, etc.), among others.

3.1 Coverage

Coverage is of particular importance in the context of somatic analyses. Although approximately 25–50 reads are sufficient for the detection of a heterozygous germline variant [33], a much deeper coverage is necessary to detect the low-frequency somatic mutations. Indeed, unlike constitutional genetics, it is not possible to confirm these mutations using Sanger sequencing, firstly due to the fact that mutations below 20% are difficult to detect using this technique, and secondly, because the confirmation is time-consuming in the context of oncohematological analyses in which rapid results are required. The mean coverage to be achieved is dependent on the library preparation and platform, the panel used and its uniformity, and most importantly, the variant allele frequencies (VAFs) that need to be detected. For instance, if the VAF to be detected is 5%, it is recommended that all the regions are covered with a minimum of 500 reads, and the mean coverage could be adopted to achieve the result. However, extremely high coverages may lead to the detection of false positives, and the most suitable compromise between these two constraints could be determined during validation. It is important that the regions which do not fulfill the coverage criteria do not contain hotspot mutations. With oncohematology panels run on Ion Torrent platforms, a mean coverage of 2000 to 3000 reads usually generates satisfactory results for the detection of mutations at 5%. Indeed, 5% of 2000 reads implies 100 mutation reads, and 5% of 500 reads implies 25 mutation reads, which is, for the majority of the positions, above the background noise.

In case of follow-up samples, a deep read coverage at the initially mutated genomic positions is essential. Indeed, after treatment, extremely small clones with mutations may persist and may be detected by using NGS. With sufficient coverage and determination of the background noise at each mutated position (e.g., through comparison with controls having a similar coverage), it is possible to detect very low residual mutation frequencies. These thresholds may be determined during validation, for example, with serial dilutions and comparisons using sensitive methods such as quantitative PCR (qPCR) or droplet digital PCR (ddPCR). Recently, the use of molecular barcodes has allowed the detection of very low-frequency mutations (<1%) with high accuracy. Molecular barcodes label each molecule within a sample individually prior to amplification, enabling the distinction of the true mutations present in the sample from the artifacts appearing at various steps of the procedure, which are unavoidable with the use of the current technologies. However, the sequencing depth required for this technology is higher than that required for conventional NGS, and the cost, therefore, increases. Moreover, the risk of identifying low-frequency mutations in healthy individuals highly increases. Therefore, the advantages and disadvantages of this technology should be carefully evaluated prior to implementing it in a diagnostic setting [31, 34].

3.2 Difficult Regions and Identification of Sequencing Artifacts

In order to ensure complete NGS analysis, challenging genomic regions including recurrent (*hotspot*) mutations have to be identified and paid special attention during validation. The genomic regions that are in general difficult to sequence, such as the GC-rich regions, repetitive regions, and homopolymers, are often associated with strand bias and poor coverage when using amplicon sequencing. These genomic regions are prone to error and often contain sequencing artifacts that have to be distinguished from the true mutations. Sequencing artifacts often have the following characteristics: they are located in difficult regions; they are low coverage regions;

they are located close to the end of the reads or in regions with strand bias; they have low VAFs; there is unbalanced coverage between the forward and reverse reads, and poor base-calling quality (e.g., Phred scores). Moreover, sequencing artifacts are recurrent within the same technology and within each individual platform, and a good approach to discard these artifacts is by comparing several samples within the same run or in different runs. Even more important is the visual inspection of the reads, which is recommended in order to detect the aforementioned issues. Even though a wide variety of well-performing bioinformatics tools are available for NGS data analysis and variant filtering, none of these tools allows to be perfectly configured for highlighting all the true mutations without detecting any artifact. Moreover, even if a variety of parameters could be adjusted during validation in order to optimize the analysis, the number of parameters is highly dependent on the software. In addition, it is not always possible to know how exactly the bioinformatics filtering and adjustments would impact the variant calling, particularly with the use of commercial software packages which provide a black box impression to the final interpreters. Therefore, visual inspection by a human eye remains a suitable approach when feasible, in order to ensure reliable variant calling. This visual inspection also allows the detection of potential alignment and nomenclature problems, particularly in the context of deletions, insertions, duplications, and delins.

In the context of AML, one of the most challenging genes is *CEBPA*. This gene is particularly GC-rich, and reliable sequencing of the entire coding sequence is difficult, especially because most of the mutations are frameshift mutations and include large insertions and deletions. Particular attention must be paid to this gene during validation; a conventional molecular technique is recommended as an alternative if no reliable sequencing is otherwise obtained. Another challenging mutation is the c.1934dupG, p.G646Wfs*12 hotspot in the *ASXL1* gene. This mutation is located in a homopolymer of 8 guanines and could be easily mistaken for a sequencing artifact and vice versa, particularly by the use of an Ion torrent sequencer. As for the *CEBPA* gene, an alternative technique, such as fragment analysis, should be considered if no reliable sequencing is obtained, since mutations in this gene are particularly frequent in myeloid disorders.

3.3 Ongoing Internal Quality Control

In order to monitor the quality of each sequencing run, it would be ideal to sequence at least one control sample with multiple representative mutations (e.g., a commercial reference DNA with low-frequency mutations) in each run. However, this is not feasible in most situations, particularly in small- or medium-sized laboratories, because of the associated additional consumable costs. Alternative measures, such as monitoring the quality parameters described above, in order to monitor the sequencing performance could, therefore, be implemented in the routine diagnostic analyses. Sequencing of well-characterized control samples containing mutations with VAFs, at regular intervals (e.g., every few months; intervals to be determined individually by each laboratory depending on the sample volume), continues to be a suitable additional method for quality control. More importantly, such control samples may be tested prior to implementing small changes such as a new version of a kit or software, or minor modifications in the standard operation procedure (SOP). In order to incorporate major changes, such as the introduction of a new panel, a new sequencing machine, or a new bioinformatics pipeline, etc., a complete revalidation is necessary.

An interesting tool available for the measurement of a potential sample mix-up is the sample IDs. Sample IDs represent a small panel containing several highly polymorphic genomic substitutions along with a sex determination tool which can be mixed with the panel to be sequenced. This allows the establishment of a single nucleotide polymorphism (SNP) profile for each patient, which further allows the confirmation of the patient identity (i.e. the same patient being sequenced) at each follow-up, even if the initial mutations have disappeared by that time.

3.4 External Quality Controls (EQA)

Besides the internal quality controls, external quality controls (EQAs) are of high importance. The participation of EQAs is mandatory in order to ensure the quality of the results and to work in an accredited manner. Three institutions have currently proposed NGS quality controls for hematological neoplasia. Stichting Kwaliteisbewaking Medische Laboratoriumdiagnostiek (SKML) offers NGS EQAs for mutation analysis in myeloid leukemias. European Research Initiative on CLL (ERIC) offers NGS EQAs for *TP53* mutation analysis in chronic lymphocytic leukemia (CLL). United Kingdom National External Quality Assessment Service (UK NEQAS) has proposed pilot schemes for myelodysplastic syndrome (MDS), AML, myeloproliferative neoplasm (MPN), and CLL. Although all of the above-listed hematology-related NGS EQA schemes are currently in the pilot phase, ISO 17043 accredited NGS somatic EQAs for hematological malignancies will be available in the near future. In the case when the EQAs for hematological disorders do not cover the particular neoplasia being tested in the laboratory, the participation in a technical EQA by testing the NGS performance could serve as a suitable alternative.

4. Reporting Content

In oncohematology, reporting of results involves several elements. As for germline genetic testing, a few of these elements should appear invariably and regularly in the oncohematology genetic report [35-37].

- Name and address of the laboratory performing the test
- The patient's identifications (complete name, date of birth, and gender)
- The sample identification (sample type and the date when the sample was taken)
- A laboratory/analysis number assigned to the sample
- The reason for the referral
- The referring clinician (name and address)
- The date of report
- Page numbers
- Signature of a Registered Clinical Laboratory Genetic Scientist
- Technical information Including:
  - a list of genes, transcripts, and regions analyzed, whenever possible
  - the technology used, along with the instrument
  - the overall and mean coverage
  - the reference genome
  - the technical limitations

The report must be brief, comprehensive, and when possible, should not exceed one page in total.

A summary of the results may be provided in a tabular format; however, the results should also be unambiguously described within the main body of the report.

The reference sequence as well as the Human Genome Variation Society (HGVS) nomenclature must be used in order to unequivocally describe the molecular genetics variants [38]. It is not compulsory to add previous gene names, although this might be useful in improving clarity among the referring clinicians.

Whenever external references (e.g., PubMed) are used, these must be mentioned either within or at the end of the report.

The application of stringent reporting measures, such as the ones described above, is recommended to achieve a higher level of uniformity and consistency across different laboratories performing similar tests.

5. Variant Classification

In oncohematology, NGS genetic testing has an increasing importance in treatment decision. Therefore, it is crucial to retain only the clinically relevant variants, and then carefully interpret these variants according to the context of the disease prior to reporting.

In the context of germline molecular genetics, the American College of Medical Genetics (ACMG) Standards and Guidelines [39, 40] recommend a 5-tier variant classification (Table 2). However, different parameters should be considered in regard to somatic variants, and a different classification system should, therefore, be applied. A Joint Consensus Recommendation for the interpretation of somatic variants in cancer [18] was published in 2017, in which a 4-tier classification was proposed (Table 2). This classification could certainly be applied to the oncohematology NGS variants; however, a less stringent categorization and a certain degree of flexibility should be maintained, as the results (e.g., the same variant) might exert different impacts according to the specific clinical context or disease group involved. Therefore, the inclusion of the variant classification (Tier I–IV) in an oncohematology report (e.g., within the table containing the results) may be considered optional. In addition, contradictions between the molecular variant classification and the clinical significance should be avoided in the conclusion. Variants classified as benign or likely benign should not appear in the report as these do not add any relevant information regarding the diagnosis, prognosis, and the predictive aim of the testing, and would rather result in a lengthy and less concise report, which is highly discouraged.

The variants identified as likely pathogenic and pathogenic at diagnosis should be clearly justified in the report. When NGS data are used for differential diagnosis, the incidence of the variant in different disease contexts should be considered. When a variant detected is compatible with the disease diagnosis even though no prognostic association is available, it should be distinctly stated in the report. Similarly, when a clear prognostic impact or resistance to a well-established treatment approach has been determined to be associated with the observed variant, a statement should be included in the report along with the reference to the corresponding published data. Large-scale studies, including risk stratification studies, should be preferred for reporting, while it is not recommended to cite smaller research studies that have not been confirmed yet. The World Health Organization (WHO) classification must be applied, whenever possible.

Table 2 Classification of Sequence Variants**:** In germline molecular genetics, a 5-tiers variant classification is recommended: Class 1–Benign variants/certainly not pathogenic; Class 2–Likely benign/Unlikely pathogenic variants; Class 3–Variants of Uncertain Significance; Class 4–Likely pathogenic variants; Class 5–Highly likely pathogenic/Pathogenic variants. For somatic sequence variants, a 4-tiers classification was proposed: Tier I–Variants of strong clinical significance; Tier II–Variants of potential clinical significance; Tier III–Variants of unknown clinical significance; Tier IV–Benign or Likely benign variants [18, 39, 40].

	GERMLINE	**SOMATIC**
Class/Tier	**5**. Pathogenic	**1.** Variants of Strong Clinical Significance
	4. Likely Pathogenic	**2.** Variants of Potential Clinical Significance
	3. Variant of Uncertain Significance	**3.** Variants of Unknown Clinical Significance
	2. Likely Benign	**4.** Benign or Likely Benign Variants
	1. Benign	

6. Databases and Other Tools

Currently, a number of databases that aim at aiding the variant classification are available.

A list of the major databases relevant to the interpretation of somatic sequence variants has been published previously [18]. Population databases (e.g., gnomAD, dbSNP, ESP) are important tools in order to classify benign and likely benign variants, whereas cancer databases (e.g., COSMIC, IARC, DoCM) generally provide information on the main tissue affected and/or relevant references if any available. Other databases (e.g., ClinVar, LOVD) may contribute to variant classification when potentially germline variants are detected.

In silico tools may be utilized to gain information regarding conservation across the species, potential splice effects, and the prediction on single nucleotide changes, although variant interpretation must not be based exclusively on the prediction provided by these tools.

Literature search and peer-reviewed articles should be regarded as the gold standard for the interpretation of NGS oncohematology variants.

7. Variant Interpretation

In oncohematology, the discovery of a specific genetic variant does not necessarily imply the inclusion of that variant in the report. The variants classified as likely pathogenic or pathogenic are the ones that have been previously reported to have a diagnostic, prognostic, and/or predictive relevance, and are, therefore, generally included in the report. However, several factors should be considered prior to the inclusion of a variant in the report, such as–

7.1 The Pre-Defined Threshold

Thresholds should be established during the test validation process to ensure the distinction of the variants detected at low VAF from the sequencing artifacts or background noise.

7.2 Sample Status

Variants detected in the samples at diagnosis and those detected at follow-up should be treated differently, as the thresholds for these two kinds of variants might differ (e.g., 10% and 2% for diagnosis and follow-up variants, respectively). Specific follow-up variants could be reported despite being below the threshold. For instance, the detection in a follow-up sample of a certain type of variant (e.g., duplications of 4 or more base pairs) at a low VAF (below the threshold) through visual examining of the read would suggest its persistence, and its reporting would, therefore, be acceptable (if prior to reporting, appropriate quality checks performed during the analysis have been successful). Reports should state whether the NGS results obtained in the follow-up cases are compatible with the patient status (remission, relapse, etc.). When a relapse or acceleration of the disease is suspected or confirmed, analysis of the complete gene panel content is recommended in order to provide a better global interpretation, as novel variants and subclones might be identified during this process.

7.3 VAF and Germline Variants

VAF may serve as a preliminary indicator of potential germline variants. Specifically, a variant at 50% [VAF] may indicate a germline heterozygous variant. Several variants present in a number of genes have been previously described as causative factors for familial MDS/AML and inherited bone marrow failure, while others have been described as the cause of inherited cancer syndromes and been associated with secondary hematological diseases [41]. In case of detection of potential germline variants that do not belong to any of the above categories (i.e., those identified within genes that are NOT causative of inherited diseases), a statement indicating the presence of a potential germline variant should be included in the report. Nevertheless, analysis of the follow-up sample, preferably in the remission state, rather than confirmation by using cultured skin fibroblasts, should be highly recommended. On the contrary, all the detected variants which could potentially be included in one of the above-stated germline categories (i.e., those which have been identified within genes causative of inherited diseases) require special attention. The age, family, and personal history of the patient are important elements for consideration. In the report, a statement indicating the detection of a potential germline variant must be included, along with recommendations for confirmatory testing. In order to confirm the presence of a germline variant, a signed informed consent must be obtained prior to performing the analysis using cultured fibroblasts from a sample of skin biopsy. Although cultured skin fibroblasts remain the gold standard for the confirmation of a germline variant in hematological malignancies, other samples (e.g., buccal swabs, saliva, hair root, and nails) might be used as well, preferably in complete remission. Nevertheless, potential contamination with leukemic cells should be considered prior to interpretation and reporting [23]. Detection of a germline variant during the confirmation analysis requires a referral to a clinical genetics department as well as communication of the results during a genetic counseling session.

In addition, the ethnicity of the patient may provide useful information for the interpretation of rare potential likely-benign variants specific to certain ethnic groups.

7.4 Clonal Hematopoiesis

Special consideration should be given to the reporting of a variant identified in specific genes (e.g., *DNMT3A, TET2, ASXL1, TP53, etc.*) when this occurs as the sole abnormality and/or in a certain disease context (e.g., suspicion of a hematological disease rather than a confirmed diagnosis). In fact, clonal hematopoiesis of indeterminate potential (CHIP) might occur in individuals without a diagnosed hematological disorder [42]. Reporting of CHIP variants with low VAFs (i.e. <5%) is not recommended generally. Nevertheless, potential CHIP variants with higher VAFs could be included in the report by ensuring a clear distinction between a potential CHIP variant and a disease-associated variant.

7.5 Variants of Uncertain Significance (VUS)

A VUS is a variant for which, even after a detailed evaluation, the impact or association with the disease is regarded as uncertain. The classification of VUS was defined when interpreting variants in the context of hereditary genetic disorders. However, in case of somatic mutation profiling in hematological malignancies, certain variants are not compatible with the original VUS classification, including variants previously not reported and clearly acquired, the ones that have been identified in genes with prognostic implications, and those which are accompanied by other mutations in the same clone or subclone. These variants are unlikely to be the driver mutations causative of neoplasia, although these could be useful in a clinical context to decipher the clonal evolution or as markers for monitoring the response to treatment. It is recommended to include these variants in the report, although by avoiding their classification as VUS.

7.6 Multiple Variants

If sequence variants are detected in several genes within the same panel, special consideration be given should be given to their prognostic value. In case of detection of the sequence variants with contradicting prognostic impacts (i.e., good vs. poor), the variants with good prognostic value tend to lose their effect. Therefore, in this context, a statement in the report regarding the good prognostic impact of these variants is considered unnecessary and redundant. In regard to the cases in which multiple variants have been detected, it is recommended to describe in the text the prognostic implication of only those variants which carry a poor prognostic value, unless the co-occurrence of certain specific variants is able to modulate the final impact.

7.7 Combined Results

If multiple genetic testing techniques are used for the analysis of the same sample, a global interpretation is recommended to be included in the report. Specifically, for the case in which conventional karyotyping, SNP array technique, and/or FISH are applied, a combined interpretation of the detected abnormalities should be provided in the report whenever possible. In fact, the overall prognostic value might vary according to the different abnormalities identified. For instance, in case of MDS, detection of a del(5q) by karyotyping and no detectable variant by

NGS would generally be associated with good response to treatment with Lenalidomide [43]; however, the concurrent detection of a *TP53* pathogenic variant would imply resistance to treatment [43]. The detected abnormalities could be organized on the basis of the applied technique, and the results generated by each of the methodologies used should be presented in the report. Nevertheless, a global conclusion is recommended. Therefore, preference should be given to combined reports and global interpretations.

7.8 Miscellaneous

- All the results must be interpreted in relation to the age of the patient, the disease type and subtype, and the disease status. Special attention should be paid on differentiating the pediatric cases from adult cases, as prognosis may differ accordingly. CHIP variants are more likely in elderly patients, while germline variants predisposing to leukemia might be more probable in young patients.
- In the case of hotspot variants only, it is recommended to state whether a specific therapeutic agent is already available or indicated. Similarly, if resistance to a well-established treatment approach is conferred by a specific variant that has been detected, that information should be provided in the report.

8. Concluding Remarks

NGS diagnostic testing in cancer has been applied widely in the case of hereditary genetics, solid tumors, and acquired oncohematology, although several differences among these fields may be highlighted (Table 3). NGS variant interpretation and reporting in oncohematology particularly remains an entangled process, in which multiple concurrent aspects are required to be considered (Figure 1). Since the crucial role of mutation screening using NGS in oncohematology has been largely demonstrated in previous studies [45], the use of this methodology is expected to increase further. Moreover, the development of novel technologies (e.g., molecular barcodes) may result in significant improvement in the detection sensitivity, and NGS mutation testing could become a powerful tool for measurable residual disease (MRD).

Currently, NGS guidelines specific to diagnostic oncohematology are, therefore, strongly required and a consensus between multiple laboratories should be of high priority.

Table 3 Diagnostic NGS panel testing in cancer: The table shows a comparison between the main elements used during the interpretation of NGS data for cancer specimens in germline, solid tumors and acquired oncohematology testing respectively [18, 39, 40, 46].

NGS Diagnostic Panel testing in Cancer	**Germline**	**Solid Tumors**	**Acquired Oncohematology**
Use / Aim	• Confirms or excludes a genetic diagnosis • Provides information to direct treatment • Provides information on carrier status and risk/recurrence assessment	• Provides additional information on the disease which can direct: diagnosis, prognosis, prediction (e.g., treatment outcome) • Identifies patients eligible for targeted treatments	• Provides additional information on the disease which can direct: diagnosis, prognosis, prediction (e.g. treatment outcome)
Testing Material	• Mainly blood	• Mainly FFPE tissue	• Mainly bone marrow
Disease / Sample /Testing Status	• Diagnosis Confirmed • Familial testing (e.g. pre-symptomatic)	• Diagnosis Confirmed or Suspected • Primary tumor • Metastasis • Liquid biopsies for monitoring treatment response	• Diagnosis Confirmed or Suspected • Relapse • Remission • Acceleration • Follow up
Variant Classification & Nomenclature	• 5 class system • HGVS	• 4 class system • HGVS	• Distinction between acquired clonal disease associated, acquired age related and (probably) germline • For acquired variants clonal disease associated, applications of a less stringent classification system • HGVS

VUS	•To be reported (unambiguous wording used to clearly distinguish VUS from other pathogenic variants)	•Rare due to hotspot targeted analysis •If any: generally reporting is laboratory dependent	•If potentially germline: to be reported (unambiguous wording used to clearly distinguish VUS from other variants) •If clearly somatic: to be reported as for the other somatic variants avoiding naming it as 'VUS'
Incidental findings	• Rare or rather N/A in panel testing	• Relatively rare due to panels targeting mainly hotspot mutations - panel dependent	• Implies germline variants including variants causing inherited cancer syndromes*
Genetic informed consent & Genetic Counseling	• Always required	• Not generally required • Required in the case of confirmation of a potential germline variant* previously detected	• Not generally required • Required in the case of confirmation of a potential germline variant* previously detected
Miscellaneous	• Additional testing methods such as MLPA or MS-MLPA may be required in order to detect larger deletions/duplications	• DNA quality may be suboptimal due to FFPE tissues • Germline genomic DNA can be analyzed in parallel, when using larger panels, in order to distinguish germline vs. somatic variants	• Some hotspot variants might be hardly detectable by NGS and additional techniques such as fragment analysis may be required • CHIP variants can be detected and interpreted according to the clinical context

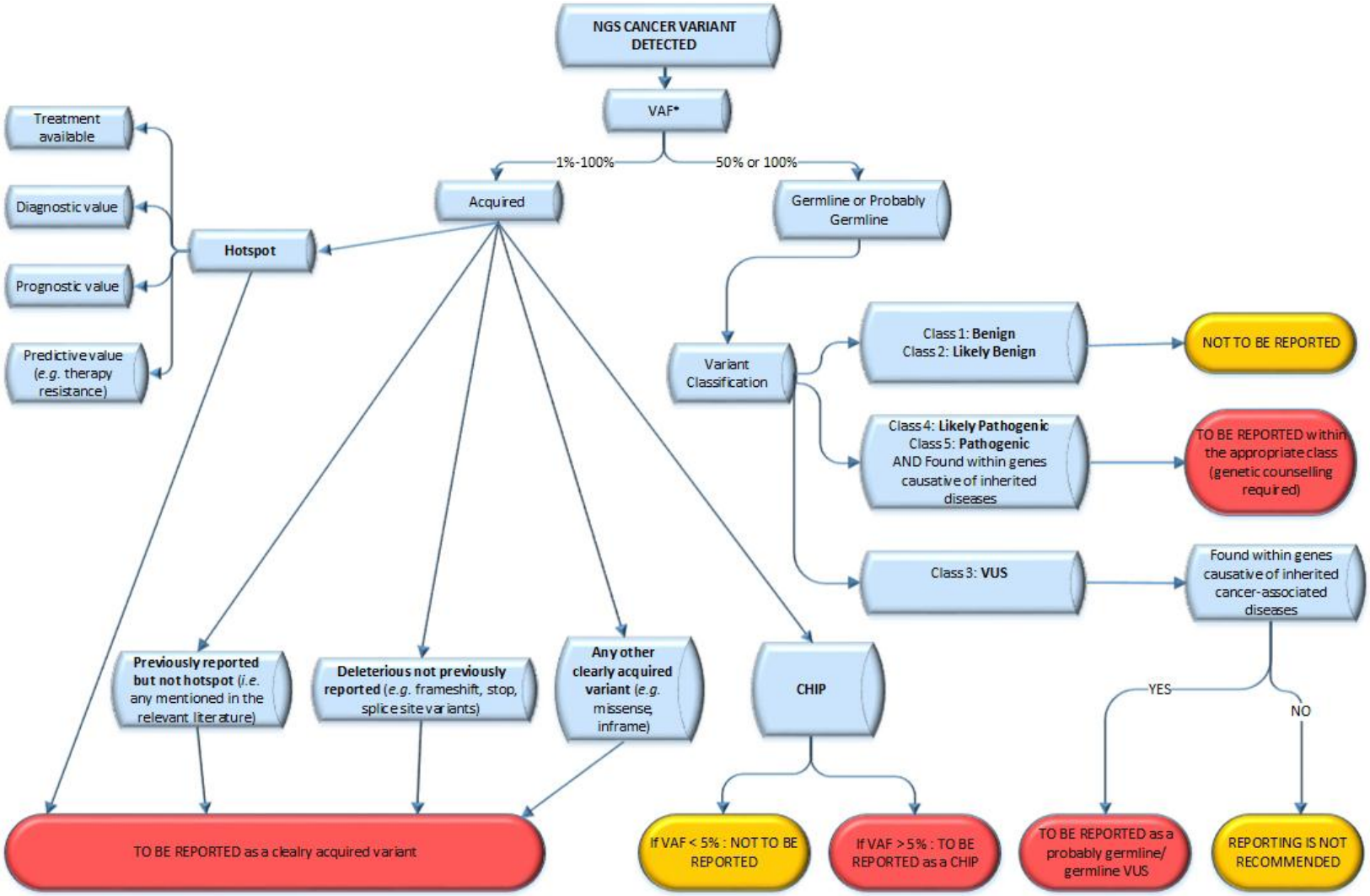

Figure 1 The chart shows the suggested workflow for NGS oncohematology variant interpretation. *It is important to note that the VAF might be either overestimated or underestimated in the case when an additional abnormality (e.g., a gain, a loss, a CN-LOH) is detected in the corresponding chromosome.

Author Contributions

JS initiated the paper, organised the work, provided ideas and critical reading, IS wrote the major part and provided the illustration and tables, FM and FRM contributed with writing and literature investigation.

Competing Interests

The authors have declared that no competing interests exist.

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