ERIC recommendations for TP53 mutation analysis in chronic lymphocytic leukemia-update on methodological approaches and results interpretation

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REVIEW ARTICLE

Chronic lymphocytic leukemia

ERIC recommendations for *TP53* mutation analysis in chronic lymphocytic leukemia—update on methodological approaches and results interpretation

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Abstract

In chronic lymphocytic leukemia (CLL), *TP53* gene defects, due to deletion of the 17p13 locus and/or mutation(s) within the *TP53* gene, are associated with resistance to chemoimmunotherapy and a particularly dismal clinical outcome. On these grounds, analysis of *TP53* aberrations has been incorporated into routine clinical diagnostics to improve patient stratification and optimize therapeutic decisions. The predictive implications of *TP53* aberrations have increasing significance in the era of novel targeted therapies, i.e., inhibitors of B-cell receptor (BcR) signaling and anti-apoptotic BCL2 family members, owing to their efficacy in patients with *TP53* defects. In this report, the *TP53* Network of the European Research Initiative on Chronic Lymphocytic Leukemia (ERIC) presents updated recommendations on the methodological approaches for *TP53* mutation analysis. Moreover, it provides guidance to ensure that the analysis is performed in a timely manner for all patients requiring treatment and that the data is interpreted and reported in a consistent, standardized, and accurate way. Since next-generation sequencing technologies are gaining prominence within diagnostic laboratories, this report also offers advice and recommendations for the interpretation of *TP53* mutation data generated by this methodology.

Introduction

Chronic lymphocytic leukemia (CLL) displays a very heterogeneous clinical behavior, therefore prognostic and predictive markers play an important role in disease management. To date, the key decision-making biomarkers in CLL are *TP53* gene defects: chromosomal aberrations of 17p13, in particular deletions spanning the *TP53* locus, and

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TP53 gene mutations, both of which are associated with adverse disease outcome due to resistance to chemoimmunotherapy [1–4].

Early studies utilizing fluorescent in situ hybridization (FISH), for the detection of cytogenetic aberrations, revealed that CLL patients carrying del(17p) have a significantly shorter overall survival compared to patients harboring other recurrent cytogenetic abnormalities, i.e., del(11q), trisomy 12, or del(13q) [5]. Inactivation of the *TP53* locus due to del(17p) is frequently associated with mutation(s) on the second *TP53* allele. However, *TP53* mutations also occur in the absence of del(17p) in about 5% of untreated patients and are associated with a poor outcome, similar to the disease course observed in del(17p) CLL patients [6, 7]. More specifically, approximately 90% of patients with del(17p) carry a *TP53* mutation; conversely, only 60–70% of patients with *TP53* mutation also harbor del(17p), as detected by FISH [8–12].

The clinical utility of *TP53* mutation analysis in CLL has been well documented by many studies [7–9, 11, 13], including findings from prospective clinical trials [6, 14, 15]

clearly showing that patients carrying *TP53* defects are resistant to chemoimmunotherapy. In this context, the advent of novel treatment options inhibiting B-cell signaling and anti-apoptotic BCL2 that proved efficacious in patients harboring *TP53* gene disruption [16–18] has brought an urgent need for accurate assessment of the *TP53* gene status in routine clinical practice with the aim of identifying those patients who would not benefit from chemoimmunotherapy, and hence should be considered for targeted agents.

TP53 gene assessment should always be performed prior to initiation of the first and every subsequent line of treatment [19]. That said, a few situations exist where *TP53* mutational analysis may not be required, e.g., when the use of p53-independent drugs is not possible due to either patient fitness or limited market access, or when the presence of a *TP53* alteration has already been documented.

The recent introduction of high-throughput next-generation sequencing (NGS) has led to the identification of *TP53* mutations with a low variant allelic frequency (VAF) —usually below the detection limit of conventional Sanger sequencing—that may be positively selected with the use of chemotherapy, ultimately leading to the expansion of an initially minor *TP53* mutant subclone into a prevalent refractory clone [20–24].

Taken together, the recent therapeutic and technological advances necessitate an update of the previously published ERIC recommendations for *TP53* mutation analysis in CLL [19], including assessment of the current methodological approaches as well as recommendations for the interpretation of the findings and the accurate reporting of results. An overview of the updated recommendations is provided in Table 1.

Procedure description

Material for TP53 mutation analysis

For most CLL patients, peripheral blood (PB) is an appropriate starting material for *TP53* mutation analysis. Nevertheless, an important factor influencing the result is the cancer cell fraction (CCF), and this is particularly relevant in cases with a low lymphocyte count ($<10 \times 10^9$/L and/or <60–70% lymphocytes in PB). This is usually evidenced in patients with predominant lymphadenopathy and few circulating clonal cells, i.e., small lymphocytic lymphoma. In such cases, material enriched with tumor cells such as bone marrow (BM) or lymph node biopsies may be an alternative option.

PB or BM should be collected in tubes containing an anticoagulant, such as EDTA or heparin, followed by mononuclear cell separation by density gradient centrifugation to enrich the lymphocyte fraction. The use of mononuclear cells might be insufficient when the specimen analyzed contains less than 60–70% lymphocytes and could lead to a false-negative result when using Sanger sequencing (Supplementary Fig. S1). In such instances, selection of CD19+ cells using enrichment techniques such as RosetteSep or MACS should be performed to yield a higher CCF. Alternatively, ultra-deep NGS, which has a much greater sensitivity level, can be performed and the VAFs corrected with respect to the CCF. Regarding tissue material, fresh/frozen material is strongly preferred. Formalin-fixed, paraffin-embedded (FFPE) tissues are recommended only when no alternative sample is available as the fixation and embedding processes may hamper the analysis, since: (i) FFPE material often contains highly degraded DNA fragments, therefore shorter amplicons are required for sequencing; (ii) the process of tissue fixation damages DNA through cross-linking, thus reducing the number of intact DNA molecules added into the PCR [25]; and (iii) DNA can be chemically modified, leading to artefactual sequencing results (particularly deamination and oxidation artifacts) [26–28]. Therefore, any variants detected in DNA samples from FFPE material should be confirmed by independent PCR and carefully verified using the recommended databases (described below) before interpreting and reporting them as mutations.

Finally, when considering the type of nucleic acid to analyze, genomic DNA is highly recommended. Analyzing RNA may result in truncating or splice site variants being missed due to nonsense-mediated RNA decay [29]. In addition, using whole-genome amplification for diagnostic purposes is discouraged as it may introduce a bias in allelic frequencies and could lead to allelic drop-out.

Region of interest

At a minimum, the sequenced region of the *TP53* gene must include exons 4–10, which corresponds to the DNA-binding domain (codons 100–300) and the oligomerization domain (codons 323–356). Sequencing of exon 10 is recommended as the frequency of mutations in exons 9 and 10 is similar or even higher in exon 10 as documented by the recent studies [30] (Fig. 1). Optimally, exons 2–11 should be analyzed to cover the entire coding region [30]. *TP53* gene profiling studies by NGS, which usually involves also exons 2, 3, and 11, have shown that variants can also occur in these exons, although their frequency is low (T. Soussi, unpublished results; Fig. 1). As each exon is surrounded by a splice donor and a splice acceptor site, sequencing of +2/−2 intronic nucleotides is required to detect variants which may impair splicing and translate to inactive proteins.

Sanger sequencing

Primer sequences, as well as the protocol for performing the PCR, are available on the International Agency for

Table 1 Overview of ERIC recommendations for *TP53* analysis

ERIC recommendation			Notes and alternatives
Patients		Sampling	Always when deciding about treatment	
Material		Type of material	Peripheral blood (PB)	Bone marrow, lymph nodes—suitable alternatives if PB lymphocyte count is <10 × 10⁹/L and/or <60–70%, e.g., in SLL/CLL. Fresh/frozen tissues are strongly preferred
		Anticoagulant	EDTA or heparin (in case of PB)	
		Cells	Mononuclear cells	When PB or BM contains <60–70% of lymphocytes, separation of CD19+ cells or using deep-NGS with low detection limit is recommended
		Nucleic acid	DNA	RNA analysis carries a risk of omitting truncating/splice site variants
		Covered region	Optimum: exons 2–11 (coding region), minimum: exons 4–10, always include splice sites (±2 intronic bp)	Variants found in introns at positions +2/−2 impair splicing
Procedure				
Sanger sequencing		Primers and PCR protocol	Available in the IARC TP53 database: http://p53.iarc.fr/ProtocolsAndTools.aspx	
		Sequencing	Both strands (forward + reverse)	
		Data analysis	Use software designed for somatic variant detection	Free web-based software available on the ERIC website
NGS		Protocol	Amplicon or capture-based approaches are applicable. DNA input should be calculated with respect to the limit of detection. Testing the integrity of DNA is recommended.	Several ready-to-use kits involving TP53 analysis are commercially available
		Sequencing	Minimum of 100 reads per each position. Number of variant reads for reliable variant calling should be at least 10.	≥99% minimum coverage percentage should be reported
		Data analysis	Use software designed for somatic variant detection. Validated minimal limit of detection should be 10% VAF.	Proper bioinformatics approach represents the most challenging part of NGS and no universal tool is currently available
Interpretation and clinical reporting	Variant description		Use HGVS nomenclature: http://varnomen.hgvs.org/ Report the cDNA and protein level including reference sequence	
	Interpretation		Check the detected variants using locus-specific database: IARC TP53 database: http://p53.iarc.fr/TP53GeneVariations.aspx or TP53 web site: http://p53.fr/	If a rare variant or variant with preserved functionality is detected: (1) Repeat the analysis by starting from PCR to exclude analytical errors (2) If the variant is confirmed, test the paired germline DNA
	Polymorphisms and benign variants		It is preferred not to include common polymorphisms and benign variants in the report to physicians	Using dbSNP for filtering out polymorphisms and neutral variants is strongly discouraged
	Limit of detection and clinical reporting		Report variants detectable by Sanger sequencing and variants present in >10% VAF if tested by NGS. Reporting variants between 5–10% VAF is acceptable only if explicitly stating that the clinical impact of minor subclonal mutations has not been conclusively documented in prospective clinical studies	There is not enough evidence for making therapeutic decisions based on the detection of mutations present in low variant allele frequency
	Report form		Template report form is available on the ERIC website	

PB peripheral blood, *NGS* next-generation sequencing, *VAF* variant allele fraction

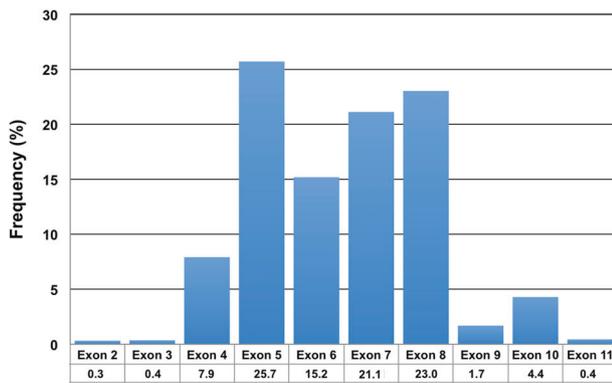**Fig. 1** Frequency of *TP53* variants detected in individual exons. Data are retrieved from the last version of the UMD_TP53 database (http://p53.fr/) and include somatic and germline mutations detected by next-generation sequencing of exons 2–11

Research on Cancer (IARC) *TP53* website (http://p53.iarc.fr/ProtocolsAndTools.aspx). This PCR protocol is adaptable and can be modified based on local experience. Bidirectional sequencing analysis is the only acceptable strategy, and the chromatograms generated by Sanger sequencing should be carefully scrutinized to ensure that somatic variants present at lower allelic frequencies are not overlooked; adjusting software settings to detect germline homozygous and heterozygous variants is not sufficient. The ERIC *TP53* Network provides the opportunity to analyze Sanger sequencing data via a web-based tool called GLASS [31]. This software was purpose-built to assist with the assessment of somatic gene variations and provides a standardized variant output as recommended by the Human Genome Variation Society (HGVS). GLASS was specifically developed to support ERIC *TP53* Network activities and is freely accessible at http://bat.infspire.org/genomepd/glass/ or via the ERIC website (http://www.ericll.org/guidance-toolstp53/).

Finally, although the relevance of pre-screening methods, such as denaturing high-performance liquid chromatography and high-resolution melting analysis is decreasing, they remain a viable and cost-effective option. That notwithstanding, in order to identify the specific variant, aberrant screening results must always be confirmed by Sanger sequencing in an independent PCR.

Next-generation sequencing

Targeted NGS can be used for the analysis of the *TP53* gene as a standalone assay or as part of a gene panel investigating several genes. Numerous commercially available ready-to-use analytical kits include the *TP53* gene, and ERIC is conducting a multi-center collaborative effort to assess and compare various pre-designed and custom gene panel technologies. Previous studies exploring the inter-

reproducibility of targeted NGS and Sanger sequencing for *TP53* analysis demonstrated very good correlation of the results, specifically showing that all variants detected by Sanger sequencing are also detectable by NGS [22, 23, 32–35]. A recent study also showed an excellent correlation between the results obtained from two different NGS platforms, namely, the Ion PGM (ThermoFisher) and the MiSeq (Illumina) [33]. In addition, NGS is capable of detecting variants below the sensitivity threshold of Sanger sequencing, even VAFs as low as <1% [20, 22, 23]. Due to the low detection limit of NGS, multiple subclonal mutations within the *TP53* gene (i.e., convergent mutations) may be detected in some patients [20, 35].

To ensure the maximum applicability and reliability of NGS, several important issues need to be addressed when establishing the methodology, as erroneous results can arise for various reasons (Table 2).

DNA input and quality

Low input and/or degraded DNA may result in false-negative results due to a sampling effect, and may also produce false-positive results as amplified errors might constitute a significant proportion of the final sequencing library [36]. The initial amount of DNA should always be calculated with respect to the required limit of detection (LOD), keeping in mind that a human cell (two alleles) contains approximately 6 pg of DNA. For reliable detection, the DNA input must ensure that the sample contains a sufficient number of variant molecules and that the variants can be distinguished from background noise. For instance, at least 10 ng corresponding to approximately 1500 cells or 3000 alleles should be used to detect variants present at 1% VAF. This is also relevant for techniques which require the starting amount of DNA to be distributed amongst individual nano-scale PCRs, e.g., the Fluidigm Access Array, RainDance Technology, or Wafergen. Although DNA isolated from PB and BM is usually of good quality, testing the integrity of the DNA by agarose electrophoresis or specialized automated electrophoresis devices is recommended (and often required) for NGS. Special attention is required when considering the quality and quantity of DNA obtained from FFPE samples due to the increased risk of false-positive as well as false-negative results.

Library preparation

Both amplicon-based and capture-based approaches are applicable. From a practical perspective, amplicon-based library preparations require much smaller quantities of input DNA and the workflow tends to be simpler and less time-intensive and labor-intensive compared to capture-based methodologies. On the other hand, hybridization capture-

Table 2 Types of NGS errors and their sources

Low quantity of DNA/degraded DNA	
False-negative result	False-positive result
Polymerase efficiency	Non-proofreading polymerase
Low/non-uniform coverage	Platform-specific sequence-dependent errors
	Using FFPE samples
Bioinformatics: Sub-optimal alignment and variant calling settings	

based approaches demonstrate better uniformity of coverage and generate fewer false-negative as well as false-positive calls as compared to amplicon-based techniques. When designing in-house primers for amplicon-based libraries, it is important to check the primer positions against potential single-nucleotide polymorphisms (SNP) and ensure that the primers can efficiently read across splice junctions. In order to establish an NGS assay with high detection sensitivity, proofreading polymerases with low error-rates are recommended. Incorporating unique molecular identifiers into the library preparation helps to distinguish errors introduced artificially during the process from true low-frequency variants and also allows for more accurate quantification (especially with PCR-based protocols) [37, 38]. Additional benchmarking studies are required to establish standard analytical methods that must then be checked for accuracy and reproducibility.

Sequencing and coverage

The required coverage should be set to ensure that the call is statistically above the background noise. Generally, the minimal coverage should not be less than 100 at any position within the regions of interest and the number of variant reads for reliable variant calling should be at least 10. The frequently reported mean or median coverage of a diagnostic panel is non-informative as uncovered regions cannot be deduced from this average value and therefore a ≥99% minimum coverage percentage is a vital requirement. Of note, the number of reads does not necessarily reflect the actual number of unique template gDNA molecules, as many reads will be duplicates generated during PCR amplification. When employing longer reads, a confident overlap (>60–70%) between the paired reads is recommended in order to avoid the introduction of false-positive results. Calling variants found in unbalanced regions with forward-reverse ratios of less than 10% (i.e., strand bias) should be avoided.

Data analysis

Multiple commercial, as well as free, software tools are available to analyze NGS data and, as the bioinformatics field is continuously evolving, no single tool is currently preferentially recommended. That said, it is of utmost importance to use a pipeline that has been optimized, and validated, for the detection of low abundance variants that must be distinguished from background error noise. Another issue concerns the accurate identification of insertions and deletions (indels), which may be missed during the alignment process, especially in the case of complex indels. Numerous indel-calling tools have been developed that often vary in the manner by which they detect indel breakpoints. Performance evaluations of indel-calling software have revealed limitations in detection; consequently, manual inspection of the data is always recommended and is particularly required for indel variants and variants close to the detection limit.

Limit of detection

LOD refers to the lowest VAF that is reproducibly detectable by the particular method under specific well-defined conditions. The LOD is a function of both the initial DNA input and the coverage achieved. The NGS assay should be established, and validated, to at least reliably identify variants detectable by Sanger sequencing and avoid false-positive calls with VAF above the Sanger sequencing detection limit (e.g., minimum LOD is 10% VAF). LOD should be set by taking into account non-uniformity of coverage across the analyzed sequence and an inconsistent error distribution. The occurrence of sequencing errors varies depending on the nucleotide position and composition and is also platform-dependent, with $C:G>T:A$ being the most frequent using Illumina platforms [39]. The error rate is also influenced by the specific sequence context (e.g., homopolymers are more prone to erroneous variant calling). The issue of detection limit and how it can influence the interpretation of findings is discussed in the following section.

Clinical reporting and interpretation of the results

Variant description

Detected variants should be described using the nomenclature devised by the HGVS nomenclature (http://varnomen.hgvs.org/) [40]. Several software programs are available to ensure adherence to standardized nomenclature (e.g., Mutalyzer; https://www.mutalyzer.nl/). Variants should be described at both cDNA and protein level, and the reference sequence number and version including the transcript and protein variant should be stated (see Supplementary Material). To standardize the output, the preferred

coding DNA reference sequence is the stable Locus Reference Genomic sequence (LRG; http://ftp.ebi.ac.uk/pub/databases/lrgex/LRG_321.xml) [30]. Transcript and protein variants 1 should be used (LRG_321t1, LRG_321p1). Special attention is warranted when annotating variants detected by NGS, especially since many bioinformatics pipelines do not fulfill the requirements for correct variant description according to the HGVS nomenclature. More specifically: (i) insertions and deletions are often not handled accurately; (ii) duplications are often misinterpreted as insertions; (iii) varying reference sequences for *TP53* within the same output are used; and (iv) the 3′ rule is not always implemented correctly. This is of particular importance for *TP53* and other genes that are oriented in the reverse direction on the chromosome. In such situations, the alignment and variant calling steps may introduce errors if aligning to the 3′ end with respect to the chromosome position rather than the coding sequence orientation.

Interpretation

Databases

The detected variant should be checked using locus-specific databases, i.e., either the IARC *TP53* database (http://p53.iarc.fr/TP53GeneVariations.aspx) [41] or the *TP53* website (UMD database; http://p53.fr/) [42]. These databases compile data from peer-reviewed literature as well as general databases, and provide information about: (i) the functional impact of all possible single-nucleotide exchanges within the coding region; (ii) the variant frequencies noted in both the somatic and the germline context; and (iii) additional relevant information, including links to other resources. The *TP53* website also provides a web-service tool called Seshat that is capable of managing files generated from NGS both in the vcf and bam formats. Seshat helps the user to: (i) check the variant nomenclature for consistency and generate a full description of each variant formatted according to HGVS; (ii) assess the pathogenicity of each variant according to general prediction algorithms and algorithms developed specifically for analyzing the *TP53* gene; and (iii) obtain functional and structural data for each *TP53* variant. Finally, variants can also be checked using the COSMIC (http://cancer.sanger.ac.uk/cosmic) or ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) databases; however, these databases are only recommended as a complementary analysis to the locus-specific databases.

Polymorphisms and neutral variants

In general, it is not recommended to include common polymorphisms and benign variants in the report to physicians. If, however, the local practice requires that these variants are detailed in the clinical report, it should be clearly indicated that the detected variant is not clinically relevant.

According to the IARC database, there are six validated exonic polymorphisms within the *TP53* gene; two are synonymous (c.108G>A: p.Pro36= and c.639A>G: p.Arg213=) and four are nonsynonymous (c.91G>A: p.Val31Ile; c.139C>T: p.Pro47Ser; c.215C>G: p.Pro72Arg, and c.1096T>G: p.Ser366Ala). The most frequent polymorphism is c.215C>G: p.Pro72Arg, where the ancestral allele C coding for proline is less frequent in the general population than the allele G [43] with latitude-dependent variations. Although the two alleles were reported to have different capabilities in inducing apoptosis and G1 arrest [44], studies analyzing the clinical impact of p.Pro72Arg and its associations with *TP53* mutations in CLL reported inconclusive results [45–48]. Reporting of the p.Pro72Arg status is therefore not recommended due to a lack of convincing evidence with regard to prognostic or clinical relevance.

Using dbSNP for filtering out polymorphisms and neutral variants is strongly discouraged as many variants listed in dbSNP exhibit loss of function and are frequently observed in cancer patients despite not being reported as pathogenic in ClinVar [49]. More specifically, of the 100 most frequent deleterious somatic variants described in the IARC database, 65 are present in dbSNP147 and only 34 are described as being pathogenic [41]. Using the data set collected within the context of the Genome Aggregation Database (gnomAD) is more accurate; however, it should be noted that several pathological variants are also listed in this database (http://gnomad.broadinstitute.org/, originally Exome Aggregation Consortium [43]).

Variants with preserved activity

If a rare variant or a variant with preserved functionality is detected, it is recommended to repeat the entire analysis, starting from the PCR step, so as to exclude analytical errors. If the variant is verified and the VAF is approximately 50%, suggesting a germline origin, it is advisable to verify the germline or somatic nature of the variant by testing patient-matched germline DNA, obtained from $CD3^+$ cells, saliva, a buccal swab or a skin biopsy (it is advised to rule out the contamination with CLL cells by flow cytometry or by testing the patient-specific IGHV rearrangement). Variants that have preserved transactivation capabilities are often found as germline and the carriers do not show any personal or family cancer-history associated with Li-Fraumeni or another cancer-predisposing syndrome. Specific examples of variants that should be considered with caution and are often inaccurately reported are c.704A>G: p.Asn235Ser or

results provided by participating labs. Examples of report forms for both Sanger sequencing and NGS are provided in the Supplementary Material and a template report form can be found on the ERIC website (http://www.ericll.org/).

Publishing and scientific reporting in the databases

It is important to distinguish between clinical reporting and reporting variants for research purposes in scientific journals. Data from publications are transferred to databases, and these databases then serve as the source of information for general use [42, 49]. For this reason, in order to prevent incorrect entries, it is essential to follow specific rules in addition to all above-mentioned basic procedures: (i) using consistent sample and patient identifiers if the data are repeatedly published, as inconsistent identification leads to redundancy in mutation databases; (ii) including the genomic coordinate and reference genome in the variant description to avoid ambiguities; (iii) listing all variants that are found in the patient including synonymous and other benign variants [55]. It is recommended to include the complete list of variants in the Supplementary Material, with appropriate description of their clinical significance. Note that if more than one variant in a patient is found, all variants should be listed. Centers following ERIC recommendations are kindly asked to mention ERIC in the "Material and methods" section of their studies and refer to this manuscript.

Concluding remarks

In CLL, inactivation of the *TP53* gene by deletion and/or mutation is strongly associated with adverse prognosis and refractoriness to chemoimmunotherapy. Detection of del (17p) and *TP53* gene mutations has become an integral part in routine diagnostics and should always be performed before deciding about treatment. Analysis of *TP53* exons 4–10 is a minimal requirement; however, ideally, the entire coding sequence, i.e., exons 2–11, should be analyzed, and this can be performed by either bidirectional Sanger sequencing or NGS. NGS also allows the parallel analysis of multiple genes and is capable of identifying variants undetectable by Sanger sequencing. That notwithstanding, NGS currently faces certain technical limitations and may lead to problems with data interpretation. The clinical importance of mutations within minor clones remains an unresolved issue and there is currently not enough evidence for making therapeutic decisions based on the presence of

mutations undetectable by Sanger sequencing. To assist the community with the implementation of *TP53* mutational analysis in a harmonized manner, ERIC created the *TP53* Network with the following objectives: regular certification of laboratories for *TP53* mutation status assessment (both for Sanger and NGS), the organization of educational events, and regular updating of recommendations for *TP53* analysis. The Network also provides tools facilitating laboratories to achieve reliable and comparable results that are accessible via the ERIC web page (http://www.ericll. org/).

Compliance with ethical standards

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