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Comparison of *TP53* mutations screening by functional assay of separated allele in yeast and next-generation sequencing in myelodysplastic syndromes

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TP53 mutations are major prognostic factors in many hematological malignancies including acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). Next-generation sequencing (NGS) has improved the detection of such mutations by identifying small mutated clones but functional method like FASAY (functional assay of separated allele in yeast) may prove interesting. We compared the detection of *TP53* mutations by FASAY and NGS in 91 patients with AML or MDS. By FASAY, 91% of assays were evaluable and 47 patients (57%) had a functional and 36 (43%) a non-functional p53 protein. FASAY could not conclude in 8 cases (9%), mainly because of poor RNA quality. No *TP53* mutation was found using NGS in 50 cases (55%), and at least one mutation was detected in 41 cases (45%). The p53 status was concordant between FASAY and NGS in 95% (79/83) of cases. The four discordances included mutations detected by FASAY only in two cases, and by NGS only in two cases. Mutations not detected by NGS consisted of insertions in intronic regions, which were not analyzed by this assay. Mutations not detected by FASAY were mutations for which the percentage of mutated allele was less than 10%, including one mutation reported as non-deleterious in the IARC database. Overall, our data suggest that FASAY is an effective and reliable method to detect *TP53* mutations in AML and MDS, which allows the assessment of the protein functionality, contrary to a sequencing approach.

1. Introduction

Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders, predominating in the elderly, characterized by ineffective hematopoiesis leading to blood cytopenias and progression to acute myeloid leukemia (AML) in approximately 30% of the cases. Among MDS, the 5q- syndrome constitutes a disease subtype defined by the presence of an interstitial deletion in the long arm of chromosome 5 and associated with better prognosis and a lower risk of transformation to AML compared to other MDS [1]. In terms of treatment, hypomethylating agents like 5-azacitidine have shown a better efficacy than conventional chemotherapy regimens in MDS [2]. Moreover, lenalidomide, an

immunomodulating agent, was shown to be particularly active in MDS with 5q- [3,4].

TP53, located on the short arm of the chromosome 17, is a major tumor suppressor gene that controls genome integrity. It encodes for p53 protein, a transcription factor involved in DNA repair, cell cycle control, apoptosis and additional functions in the cell fate like metabolism, aging, and cell stemness. *TP53* mutations occur in 5–10% of MDS and AML, especially in cases with complex karyotype, often including 17p deletion (i.e. loss of the wild-type *TP53* allele) [5] and in the 5q- syndrome, where they are found in about 20% of the cases [6]. Interestingly, the 5q- syndrome is a model of ribosomopathy, like Blackfan Diamond anemia, with haplo-insufficiency for about 40 genes including *RPS14* [7,8] leading to activation of the p53 pathway and p53-induced apoptosis of the erythroid cells. Furthermore, a mouse model of 5q- syndrome crossed with p53^{-/-} mouse does not develop the 5q- syndrome phenotype, illustrating the importance of p53 pathway in this disease [9].

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The presence of *TP53* mutation is associated with poor prognosis in AML and MDS, even in patients with complex karyotype, where it remains an independent poor prognostic factor [10]. Furthermore, *TP53* mutations in AML and MDS are associated with poorer response to chemotherapy [11], 5-azacitidine [12,13], allogeneic SCT [14] and, in the case of MDS with isolated del(5q), to poorer response to lenalidomide (especially cytogenetic response) [6,15,16]. Therefore assessing *TP53* mutations in AML and MDS appears important in routine practice to refine prognosis and to better predict resistance to conventional treatments.

The analysis of *TP53* mutational status is important for AML, high-risk and low-risk MDS. Nonetheless, there is no reference method to screen *TP53* mutations, which are largely distributed in the DNA binding domain of the protein. However, some recommendations on *TP53* mutation analysis in the context of chronic lymphocytic leukemia (CLL) are available and could guide the detection in other hematological disorders [17]. Immunohistochemistry (IHC) has been widely used as a surrogate for detection of *TP53* status and detects the p53 protein accumulation in a cell, often but not constantly caused by *TP53* mutation. On the other hand, direct sequencing using the Sanger method allows the identification of the mutation but with a sensitivity of only 15–20% of mutated allele.

Functional assay of separated allele in yeast (FASAY) is a functional method that detects *TP53* mutations by assessing p53 transcriptional activity in yeast [18]. This RNA-based method has a detection limit of approximately 10% but evaluates the function of the p53 protein with subsequent identification of the defect by sequencing. It has been largely used by our group and others in solid tumors, especially breast carcinoma [19,20], but less often in hematological malignancies. Finally, over the last few years, the development of next-generation sequencing (NGS) technologies has revolutionized our ability to analyze cancer genomes. Importantly, the detection of low mutant allele burden can now be achieved by NGS assays.

In this study, we investigated the presence of *TP53* mutations in patients with high-risk MDS or AML, or low-risk MDS with del(5q) with two different approaches, FASAY and NGS, and we compared the results obtained with both methods.

2. Material and methods

2.1. Patients and samples

We studied a cohort of 91 patients diagnosed with MDS in six French centers and composed of 24 high-risk MDS or AML patients already part of a previous publication [12], and 67 lower-risk MDS with del(5q).

For all patients, bone marrow samples at diagnosis or during disease follow-up were available. All patients provided written informed consent.

2.2. Samples preparations and qualification

DNA and RNA were extracted by phenol–chloroform methods from mononuclear bone marrow cells collected without specific RNA conservation recommendations, quantified by Nanodrop spectrophotometer and quality was estimated by agarose gel electrophoresis.

2.3. FASAY analysis and direct sequencing by Sanger method

TP53 status was assessed on RNA by the functional analysis of separated alleles in yeast (FASAY) method in the molecular oncology unit of St. Louis Hospital. This assay evaluates the transactivation activity of p53 on a p53-responsive promoter stably

integrated in the yeast genome, as initially described [18]. Briefly, RNA was reverse transcribed in cDNA using Random Hexamer and Superscript II, *TP53* transcripts were amplified by polymerase chain reaction (PCR) (exon 4–10) and cotransfected with the Gap repair plasmid in yeast [19]. Yeast colonies transformed with wild-type or mutated *TP53* sequences appear as white and large or red and small, respectively. *TP53* status was considered non-functional when more than 10% of the yeast colonies were red. Analysis of the split version of the test was performed to confirm this result and to localize the defect in the 5' or 3' part of the gene. The detection limit of this technique is 10–15%. Finally, the *TP53* defect was identified by direct Sanger sequencing of the FASAY PCR product if the percentage of red colonies was over 40% or of the cloned DNA from red yeast colonies if the percentage of red colonies was low (<40%).

2.4. NGS analysis of *TP53*

NGS analysis was performed in the hematology laboratory of Lille University Hospital using the GS Junior System (Roche, Meylan, France) and the *TP53* assay-on-demand from the Interlaboratory RObustness of Next-generation sequencing (IRON) study phase II. *TP53* exons 4–11 were amplified from genomic DNA using eight primer pairs including a molecular identifier barcode sequence. Information on primer sequences and the detailed protocol can be provided upon request.

Data analysis and variant detection was performed as previously described by Kohlmann et al. [21]. The detection limit of this assay was 1–2% of mutant allele.

3. Results

Ninety-one patients were analyzed, including 12 AML, 12 high-risk MDS and 67 low-risk MDS with del(5q).

3.1. p53 status evaluated by FASAY and NGS method

FASAY technology was performed on the 91 RNA samples available and 83 yeast assays were evaluable (91%), while the remaining eight patients had inconclusive results, which represent an acceptable result regarding the sample collection conditions. Among the 83 evaluable patients, 47 (57%) had a functional and 36 patients (43%) a non-functional FASAY (Table 1). In order to identify the alteration highlighted by the transcriptional tests, Sanger sequencing of cDNA (if % red colonies was >40%) or cloned DNA from red yeast colonies was performed and *TP53* mutations could be detected in 35 cases. The remaining single case presented no mutation but lead to the identification of the transcriptional deficient β -isoform of p53 (UPN 2, Table 2).

NGS was performed on the corresponding DNA sample of all 91 patients: 50 patients (55%) were wild-type for *TP53* and 41 (45%) had a *TP53* gene mutation (Table 1). In the 41 mutated cases by NGS, the median proportion of mutated allele was 28% (range 2–99%), including a median of 72%, 35%, 14% in AML, higher risk MDS and lower risk MDS with del(5q), respectively. Finally, by combining results, 42 of the 91 (46%) patients had at least one *TP53* gene mutation with a total number of 55 mutations identified (Fig. 1).

Table 1
Comparison of patients analyzed with FASAY and NGS methods.

		NGS	
		MUT	WT
FASAY	Non-functional	34	2
	Functional	2	45
	Inconclusive	5	3

Table 2
Detailed results of discordant cases between FASAY+Sanger and NGS.

UPN	FASAY (% of red colonies FASAY/spli5'/split3')	Mutation by Sanger sequencing	Mutation by NGS	% of mutated alleles NGS	Number of mutations in IARC database	% of this mutation among 918 TP53 mutations in the hematopoietic system in IARC database
1	36/4/29	Intron 8 insertion: p.A307Gfs*31	WT	NA	0	0
2	20/1/29	Intron 9 insertion: p53β isoform	WT	NA	NA	NA
3	3/ND/ND	WT	p.R248Q	6	942	8.93
4	8/2/3 ^a	WT	p.P222S	3	3	NR
5	98/92/7	Exon 4 deletion: p.G59Tfs*44	p.M246V	10	61 (M246V)	0.65 (M246V)

FASAY result (x/y/z): % of red colonies in the global test/% of red colonies in the 5' split version/% or red colonies in the 3' split version.
ND, not done; NA, not applicable; NR, not reported.

^a In that case SPLIT test was performed since the result of the global test showed a result close to the positivity rate.

Because eight patients had inconclusive FASAY (Supplementary Table 1), comparison between the two methods was possible in 83 fully evaluated patients. Results obtained with functional assessment of p53 by FASAY and TP53 mutational status assessed by NGS were concordant in 79/83 (95%) cases. TP53 status according to WHO classification (AML, high risk or low risk MDS) is presented in supplementary data (Fig. S1).

3.2. Comparison between FASAY followed by Sanger sequencing and NGS methods

Among the 83 FASAY/NGS analyzed cases, five results were not fully concordant for the alteration identified with both methods (Table 2). In two cases (UPN 1 and 2), FASAY was non-functional but no mutation was found by NGS on genomic DNA. In both cases, Sanger sequencing revealed insertions of intronic regions (part of intron 9 in one case and intron 8 in the other) in the TP53 mRNA, reflecting splicing defects not explored by the NGS design but clearly identified on RNA by FASAY. UPN2 was of particular interest and presented a non-functional FASAY without identification of a mutation on the cloned TP53cDNA, but identification of the transcriptional deficient p53β isoform. In two other cases (UPN 3 and 4), a mutation was detected by NGS without functional alteration on FASAY, corresponding to point mutations for which the percentage of the mutated alleles was less than 10% (3% and 6%, respectively). Nevertheless, the p.P222S mutation identified in UPN 4 was reported to present a super transactivating phenotype with a neutral effect in the IARC database. This may explain why it was not

detected by FASAY. Finally, in UPN 5, classified as mutated by both methods, the mutation detected was different: FASAY followed by Sanger sequencing identified a deletion in exon 4 leading to a STOP codon on amino-acid 147, whereas a point mutation (M246V) at low frequency (10%) was identified by NGS. In that case, migration of the TP53 cDNA amplicon on agarose gel electrophoresis showed a shorter fragment concordant with presence of a deletion (data not shown). Consequently, this patient may have had two mutations: a deletion of exon 4, not detected by NGS and a point mutation, not detected by FASAY+Sanger because of a low percentage of mutated alleles.

Finally, in the 34 concordant mutated cases, at least the highest frequency mutation could be detected by both methods. The high sensitivity of the NGS technology was able to detect multiple TP53 mutations in nine patients with mutations at low frequency (2–11%) (Table 3). Surprisingly, the FASAY followed by Sanger sequencing was able to detect 14/19 (73.7%) of these multiple mutations, even at a frequency as low as 5% (Table 3). Occurrence of several mutations in patients with an initial diagnosis of low-risk MDS with del(5q) has been more carefully analyzed (manuscript in preparation).

4. Discussion

In this work, we analyzed the TP53 mutational status in 91 patients with either high-risk MDS or low-risk MDS with del(5q) by two methods: the FASAY in combination with Sanger

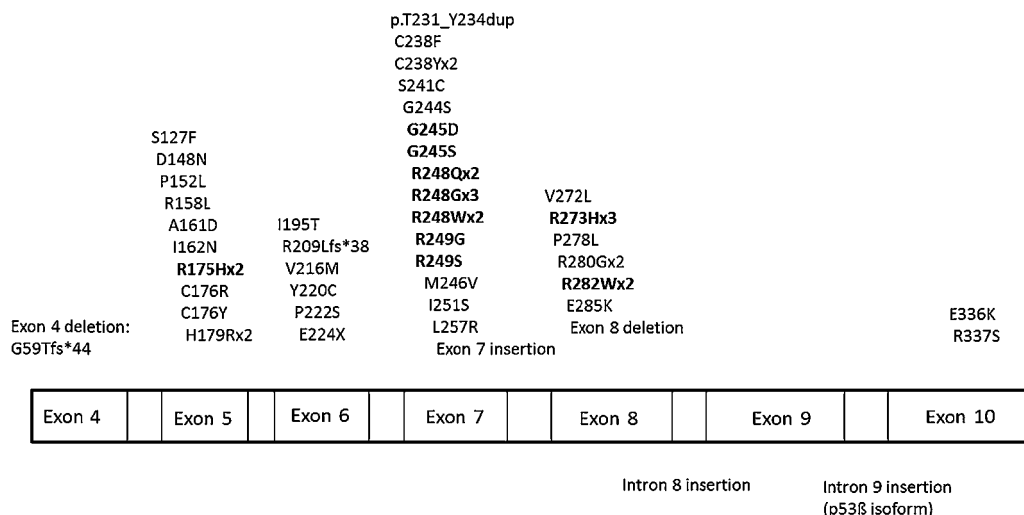


Fig. 1. Location of TP53 mutations identified in this study.

Table 3

Detailed results of patients with several mutations. NR, not reported.

UPN	FASAY	Mutation by Sanger sequencing	Mutation by NGS	% of mutated alleles NGS	Number of mutation in IARC database	% of this mutation among 918 TP53 mutations in hematopoietic system in IARC database
6	55/30/18	p.R175H	p.R175H	11	1210	4.79
		Not detected	p.C238Y	2	89	0.65
		Not detected	p.G245D	3	162	0.33
7	75/58/16	p.C176R	p.C176R	15	15	NR
		Not detected	p.G245S	8	454	1.74
8	79/43/68	p.V216M	p.V216M	28	80	0.22
		p.P152L	p.P152L	8	87	0.22
		Not detected	p.R209Lfs*38	28	0	NR
9	54/4/37	p.V272L	p.V272L	34	52	NR
		Not detected	p.T231.Y234dup	19	0	NR
10	85/6/60	p.C238F	p.C238F	12	45	0.11
		p.R273H	p.R273H	13	851	2.61
11	29/3/34	p.R282W	p.R282W	8	602	1.2
		Not detected	p.C238Y	2	89	0.61
12	44/4/33	p.R249G	p.R249G	25	53	NR
		p.S127F	p.S127F	5	32	NR
13	27/1/42	p.R280G	p.R280G	10	45	0.33
		p.R248G	p.R248G	8	26	0.22
14	72/51/43	p.A161D	p.A161D	36	23	NR
		p.S241C	p.S241C	32	35	0.44

sequencing presenting a sensitivity around 10–15%, and a NGS assay with a sensitivity around 1–2%.

NGS results could be obtained in 100% of analyzed cases while the more tricky analysis of the RNA by FASAY was possible in 91%, without specific sample collection recommendations. Collectively, by FASAY and/or NGS, 42 patients (46%) presented a *TP53* alteration with a total of 55 mutations. *TP53* alterations were predominantly missense mutations (50/55) but we identified also insertion/deletion in 5/55 cases.

Results and subsequent comparison between the two methods could be obtained for 83 patients with an excellent concordance of 95% (79/83). The five discordant cases were carefully analyzed. The two FASAY false negative cases (2.3%) could be explained by the presence of the mutated alleles at low percentage (3% and 6%), thereby only detectable by NGS. But one case (p.P222S) may also have been explained by the fact that the mutation is described in the IARC database (<http://p53.iarc.fr/>) as a non-deleterious (functional classification based on SIFT program) mutation that does not inhibit the transcriptional activity of the protein. This functionality is directly addressed by the FASAY test whereas NGS data require further functional interpretations. The one NGS false negative case, consisting of an intron 8-insertion, was not detectable because intronic regions were not analyzed by the assay. The discrepancy in UPN 5, despite a correct classification of the patient as *TP53* deficient, also illustrates the aforementioned pitfalls of the two technologies: less sensitivity for FASAY and less specificity of NGS for ins/del mutations.

Finally, UPN 2 is of particular interest because the non-functional FASAY lead to the identification of the p53 β isoform, undetectable by the NGS technology. This case cannot strictly be considered as mutated because the p53 β isoform exists in normal tissues [22], and thus could be considered as a false positive case of the FASAY technology, which was however correctly identified with the subsequent Sanger sequencing. Nevertheless, presence of this p53 isoform could have clinical implications that should be explored. Indeed, the p53 protein was the first isoform identified in the 80s but to date, at least twelve p53 protein isoforms have been described [23]. These isoforms are differentially expressed in human cancer types and the imbalance between p53 isoforms expression could have clinical consequences. Recently, alterations in the SRSF3 splicing factors were shown to induce these alternatively spliced p53 β isoform with promotion of cellular senescence

[24]. This finding could be particularly interesting in the context of MDS where somatic mutations of splicing factors have already been described and seem to have prognostic significance [25].

Interestingly, in our work, several mutations could be detected in 9/83 patients (total of 19) by NGS. Among these mutations, 73.7% were also detected by FASAY followed by Sanger sequencing despite the fact that the additional mutations were present at low rate (from 5% to 15%). This sensitivity can be further improved by extended cloning and sequencing of red yeast colonies, each colony representing a mutated allele.

It has previously been shown that none of the existing methods of *TP53* gene analysis allowed detection of all *TP53* abnormalities, especially for the two methods used in our study [17]. Due to the increased interest in the assessment of *TP53* status in hematological malignancies, an accurate validated method is needed. The most suitable technology must be evaluated not only according to the technical feasibility but also to cost, and clinical context.

Immunohistochemistry (IHC) has been used for a long time to detect *TP53* mutations easily. This method is based on the fact that the *TP53* mutated protein accumulates in the cell because no longer degraded by MDM2. Nevertheless, mutations leading to the absence of the p53 protein are undetectable by IHC and represent, 6.06% of *TP53* mutations in MDS (IARC database, January 2015). Also, normal p53 accumulates physiologically in stress, inflammatory and senescent situations [26]. Thus the detection of p53 accumulation by IHC constitutes only an indirect, non-constant, consequence of a *TP53* mutation [27]. Especially in the del(5q) syndrome, the disease related p53 pathway activation is likely to impede the correct evaluation of the p53 status by IHC alone.

The FASAY method is a fast and cheap method, described in 1995, which does not require specific and expensive instrumentation. It must be followed by Sanger sequencing if one wants to characterize the alteration. In our study, FASAY combined to Sanger sequencing appears to be a reliable method to detect *TP53* mutations in MDS, as also recently described in the CLL context [28]. The major limitation of this technology is the need of good quality RNA. Nevertheless, high RNA quality can easily be obtained by blood or bone marrow sample collection with specific reagents stabilizing RNA and avoiding its degradation [29]. The FASAY method has a lower sensitivity compared to NGS but, in our study, the percentage of red yeast colonies (mutated alleles) was often high and allowed an accurate evaluation of *TP53* status. Indeed, 67 patients

had del(5q)MDS and strong evidence showed that haploinsufficiency for the long arm of chromosome 5 leads to p53 pathway activation and increased levels of *TP53* mRNA and p53 protein in this MDS subtype [9,30–32]. This could explain why, on RNA, *TP53* mutations were easily found by FASAY followed by Sanger sequencing with quite high percentage of red colonies (median of red colonies = 55%) even when these alterations were detected on DNA at very low level by NGS (median percentage of mutated alleles = 14%).

The main advantage of FASAY is the identification of the *TP53* defect by its functional impairment and the exploration of abnormalities only present in RNA.

With the development of the new sequencing technologies, highly sensitive methods of sequencing are now available in routine laboratories. It allows the detection of many mutations at low and very low (deep sequencing) rate in all types of diseases, especially in cancers. It is usually performed on DNA, easily obtained, but needs large laboratories with heavy initial cost for instrumentation but becomes cost-effective if multiple samples are explored together.

In our hand, a combination of different methodologies could help to circumvent known pitfalls of each technique. In a few cases, FASAY followed by Sanger sequencing was helpful to identify some mutations by NGS, such as insertions and deletions. In a few other cases, NGS allowed the detection of some point mutations, which were present at a very low rate. Finally in 2015, while the cost of NGS analysis for *TP53* mutations is evaluated at around 200 dollars when performed alone (and around 1000 dollars when combined to analysis of the 30 main other genes involved in MDS and AML) the cost of the FASAY technique is around 20 dollars (prices including reagents only), with 10 dollars additional costs if completed by Sanger sequencing.

Taken together, our data suggest that FASAY in combination with Sanger sequencing is a reliable and cost-effective method to detect *TP53* mutations in MDS, especially in del(5q) syndrome. Notably, it allows the detection of insertions/deletions currently not well detected by NGS and gives a functional information, which can be helpful in clinical practice.

Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.leukres.2015.07.001>.

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