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Exploring p53 protein expression and its link to *TP53* mutation in myelodysplasia-related malignancies—Interpretive challenges and potential field of applications

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Aims: *TP53* alterations have a significant prognostic effect in myeloid neoplasms. Our objective was to investigate the *TP53* gene mutation status, p53 protein expression and their relationship in dysplasia-related myeloid neoplasms with varying levels of myeloblast counts.

Methods and results: A total of 76 bone marrow biopsy samples with different blast counts were analysed. Total and strong (3+) p53 expression was determined. Dual immunohistochemical staining was performed to determine the cell population associated with p53 expression. NGS analysis was performed using the Accel-Amplicon Comprehensive *TP53* panel. Both p53 expression and *TP53* VAF showed a significant correlation with the myeloblast ratio ($P < 0.0001$); however, p53 expression was also present in other cell lineages. The VAF value exhibited a significant correlation with p53 expression. A high specificity (0.9800) was observed for *TP53* mutation using the ≥ 10% strong (3+) p53 cut-off value, although the sensitivity (0.4231) was low.

Conclusions: Strong (3+) p53 expression using a ≥ 10% cut-off value accurately predicts *TP53* mutation but does not reveal the allelic state. The p53 expression is significantly influenced by myeloblast count, and histological interpretation should consider the presence of intermixed non-neoplastic marrow cells with varying physiological p53 expression.

Keywords: bone marrow, myeloblast, myelodysplasia, p53 expression, *TP53* mutation

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Abbreviations: AA, amino acid; AML, acute myeloid leukaemia; AML-MR, acute myeloid leukaemia with myelodysplasia-related changes; CCSS, comprehensive cytogenetic scoring system; DNA, deoxyribonucleic acid; IHC, immunohistochemical; IPSS, International Prognostic Scoring System; IPSS-R, Revised-International Prognostic Scoring System; MDS, myelodysplastic neoplasm; MDS-biTP53, myelodysplastic neoplasm with bi-allelic TP53 inactivation; MDS-IB, myelodysplastic neoplasm-increased blast; MDS-LB, myelodysplastic neoplasm-low blast****; MN, myeloid neoplasms; MN-pCT, myeloid neoplasms post-cytotoxic therapy; NGS, next-generation sequencing; SNV, single nucleotid variants; VAF, variant allele frequency; WHO, World Health Organisation.

Introduction

Myelodysplastic neoplasm (MDS) is a group of clonal haematopoietic stem cell disorders, defined by cytopaenias and morphological dysplasia, leading to progressively ineffective haematopoiesis and an increased risk of acute myeloid leukaemia (AML).[1]

MDS is a heterogeneous group of preleukaemic diseases with varying prognoses, clinical phenotype and responses to treatment. It is crucial to use optimised risk stratification in individual patients for effective treatment strategies. To achieve this, different properly validated prognostic scoring systems, such as the International Prognostic Scoring System (IPSS) or the Revised-IPSS (IPSS-R), are used worldwide.[2,3]

Apart from recurrent cytogenetic abnormalities, oncogenic mutations have been detected in 78% of patients with MDS or closely related myeloid neoplasms.[4] Among the most frequently mutated genes in MDS, each plays a role in distinct cellular processes.[5–7] Certain mutations in MDS have strong associations with clinical phenotypes and outcomes, making them valuable prognostic biomarkers independent of conventional scoring systems.[7,8] Among these mutations, *TP53* aberrations hold particular significance in MDS, as they are detected in 7–11% of patients. *TP53* mutations are often linked to abnormalities of chromosome 5q, high cytogenetic complexity or monosomal karyotypes.[9,10] *TP53* mutations are also frequently found in certain AML subgroups, such as AML with myelodysplasia-related changes (AML-MR).[11] These mutations are well-established adverse risk factors in both AML and MDS.[12,13] Patients with *TP53* alterations exhibit lower rates of complete remission, increased relapse rates and inferior overall survival following intensive treatments.[14–16] Similarly, those with *TP53* mutations experience shorter remission durations and inferior overall survival after non-intensive treatments.[17,18] It has been described that cases with prior cytotoxic therapy tend to carry *TP53* alterations.[1] Similar to solid neoplasms, various alterations of the *TP53* gene have been identified in myeloid neoplasms. These alterations include allelic losses at the cytogenetic level, as well as molecular mutations, insertions and deletions. These aberrations can manifest as either heterozygous with a remaining wild-type allele or in a hemi-/homozygous state.[19,20]

In the fifth edition of the World Health Organisation (WHO) classification, MDS is divided into two main subcategories: those having defining genetic abnormalities and those that are defined morphologically. MDS with bi-allelic *TP53* inactivation (MDS-biTP53) falls into the group of MDS with defining genetic abnormalities. MDS-biTP53 is defined as a myeloid neoplasm with cytopaenia, dysplasia and fewer than 20% blasts or 30% erythroblasts, characterised by two or more *TP53* mutations or one *TP53* mutation and concurrent evidence of *TP53* copy loss or copy neutral loss of heterozygosity.[1] It has been established that cases harbouring a mono-allelic *TP53* mutation manifest analogous behaviour to wild-type cases, while those exhibiting multi-hit/bi--allelic alterations demonstrate markedly diminished prognostic outcomes.[21] The International Consensus Classification defined a distinct category named 'myeloid neoplasms with mutated *TP53*' encompassing MDS, MDS/AML and AML cases, according to the blast percentage. This classification also highlights the importance of detection cases with multi-hit *TP53* alterations.[22]

Previous studies have examined the potential prognostic significance of p53 overexpression defined by immunohistochemistry and its relationship with *TP53* mutation. The evidence suggests that increased p53 labelling is linked to a higher risk of AML evolution, reduced overall survival, elevated bone marrow blast count and poor risk karyotype.[23–26] However, these studies vary in terms of the staining intensity and cut-off values used. Remarkably, there is only limited information regarding the topographic characteristics of p53 expression in different bone marrow lineages.[27]

This retrospective study aimed to analyse p53 expression in bone marrow samples with dysplasia, both in cases of MDS and dysplasia-related AML groups. We examined the correlation between *TP53* mutation and p53 immunopositivity by employing various cut-off values for p53 expression. Additionally, we focused upon understanding the relationship between p53 expression, *TP53* mutation and myeloblast count. Double immunohistochemical (IHC) stains were used, which allowed us to define precisely the expression of p53 in different bone marrow cell lineages. Through these techniques, we aimed to gain a deeper understanding of the role of p53 in the bone marrow context and its potential implications for disease progression and patient outcomes in dysplasia-related conditions.

Materials and Methods

PATIENTS AND SAMPLES

In this retrospective study, 46 adult cases of myelodysplastic neoplasm (MDS) and 26 adult cases of AML were evaluated. All cases exhibited dysplastic

morphological changes in at least one bone marrow cell lineage. Additionally, four adult cases without evidence of a clonal alteration were selected as controls. A total of 76 formalin-fixed paraffin-embedded bone-marrow biopsy samples were collected from the archives of the Department of Pathology, Medical Center, University of Debrecen. The patient's average age was 64.2 years (ranging from 25 to 90 years), and the female-to-male ratio was 42 of 34. The patients were managed and treated at the Department of Haematology, Medical Center, University of Debrecen. The latest edition of the WHO classification of haematolymphoid tumours was used for reclassifying the cases. The study protocol was approved by the respective Institutional Ethical Review Board for human subjects (IRB reference number: 60355-2/2016/EKU and IV/8465-3/2021/EKU).

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Bone marrow biopsies were routinely processed, fixed in formaldehyde (3.6% in phosphate buffer) for 1 day, followed by decalcification [ethylenediamine tetra-acetic acid- tromethamine (EDTA-TRIS) buffer at a concentration of 0.1 g/ml], and then embedded in paraffin wax; 4 μm slides were deparaffinised and used for subsequent stainings.

Retrospective p53 IHC analysis was performed using the Do-07 clone Dako antibody (Agilent Technologies Company, Santa Clara, CA, USA), following the manufacturer's instructions. Two independent pathologists evaluated the percentage of positive cells (0–100%) and the intensity of nuclear staining. The intensity of staining ranged from 0 to 3: 0 for negative, 1+ for weak, 2+ for moderate and 3+ for strong expression. The total percentage of p53-positive cells (weak to strong) and the percentage of cells with strong (3+) p53 expression were assessed. Three cut-off values (1, 5 and 10%) for strong (3+) nuclear expression were correlated separately with the mutational status. Accuracy of strong (3+) and total p53 expression for prediction of *TP53* mutation status were also analysed. Specificity, sensitivity, positive predictive value and negative predictive value were determined using different cut-off values. To analyse the p53 expression of different bone marrow cell populations, double IHC stainings were performed sequentially using the EnVision FLEX/HRP system. CD71 and CD34 antibodies were used in combination with p53 immunohistochemistry. Methyl-green counterstaining was performed for double staining.

DNA ISOLATION

DNA extraction was carried out using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany). DNA concentration was measured using the Qubit dsDNA HS assay kit in a Qubit 4.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions.

NEXT-GENERATION SEQUENCING

Following genomic DNA enzymatic fragmentation, libraries were created using the Accel-Amplicon Comprehensive *TP53* panel (Swift Biosciences, Ann Arbor, MI, USA). Sequencing was performed using the MiSeq System (MiSeq Reagent kit version 3, 600 cycles; Illumina, San Diego, CA, USA), as per the MiSeq instruction manual. Captured libraries were sequenced in a multiplexed fashion with a paired-end run, obtaining 2 × 150 base pair (bp) reads with at least ×250 depth of coverage. The coverage ranged between 250 and 69 361, with a median of 4075 and a mean of 13 769. The trimmed fastq files were generated using MiSeq reporter (Illumina). NextGENe software version 2.4 (SoftGenetics, State College, PA, USA) was used to analyse the raw sequence data for the presence of single-nucleotide variants (SNVs) and small insertions and deletions (indels). The human reference genome GRCh37 (equivalent UCSC version hg19) was used for alignment. The NGS sequencing strategy is suitable to detect splice site mutations. A 5% variant allele frequency (VAF) was used as a cut-off.

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism version 5.0.3 for Windows (GraphPad Software, San Diego, CA, USA). *P*-values below 0.05 were considered statistically significant.

Results

CASE DISTRIBUTION BASED ON MYELOBLAST COUNT, P53 EXPRESSION AND TP53 STATUS

Considering myeloblast counts, the study included 26 cases of AML with ≥ 20% bone marrow myeloblasts, 12 cases of MDS with an increased blast count (5–19% bone marrow myeloblasts) and 34 cases of MDS with a low blast count (< 5% bone marrow myeloblasts). The cases were re-evaluated and classified

according to the diagnostic criteria of the 2022 WHO classification of haematolymphoid tumours (Table 1, Figure 1).

Table 1. Distribution of cases (n = 76) according to different parameters

World Health Organisation diagnosis according to 5th edition, 2022	
AML-MR	23
MN with bi-allelic (or multi-hit) TP53 alterations	2
MN with low blasts and 5q deletion	3
MN with increased blasts (morphologically defined)	8
MN with low blasts (morphologically defined)	23
Hypoplastic MDS (morphologically defined)	4
MN-pCT-AML	3
MN-pCT-MDS with increased blasts	2
MN-pCT-MDS with low blasts	4
Reactive, control	4
Sex of patients	
Male	34
Female	42
TP53 mutational status by NGS	
TP53 wild-type	49
TP53 mutated	27
TP53 mutated with multiple mutations	8
p53 expression by immunohistochemistry	
p53 total expression ≥ 10%	25
p53 strong (3+) expression ≥ 1%	32
p53 strong (3+) expression ≥ 5%	17
p53 strong (3+) expression ≥ 10%	12
Karyotype according to CCSS	
Good	38
Intermediate	6
Poor	2
Very poor	14
No data	13

CCSS, Comprehensive Cytogenetic Scoring System; NGS, next-generation sequencing; MDS, myelodysplastic neoplasm; MN, myeloid neoplasms; AML-MR, acute myeloid leukaemia myelodysplasia-related; MN-pCT, myeloid neoplasms post-cytotoxic therapy.

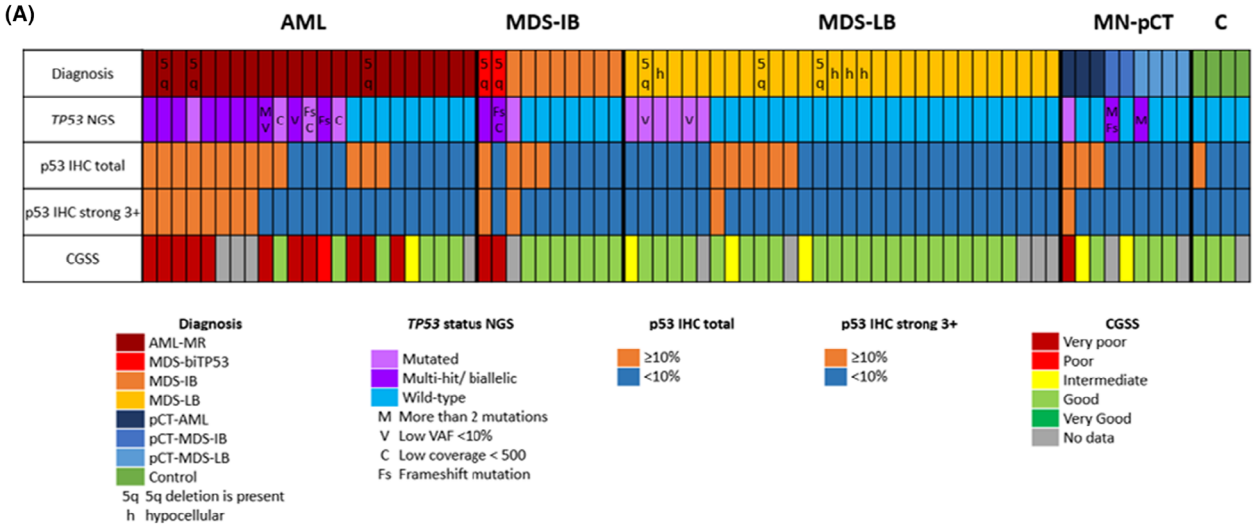
Different cut-off values for p53 expression were defined, including ≥ 10% total p53 expression, ≥ 1% strong (3+) p53 expression, ≥ 5% strong (3+) p53 expression and ≥ 10% strong (3+) p53 expression. The correlation between the total percentage of p53-positive cells, assessed by two independent pathologists, was found to be highly significant (Spearman's $r = 0.7293$, $P < 0.0001$). An inter-reader agreement was nearly perfect using the ≥ 10% cut-off values for strong (3+) p53 expression, with a Kappa-value of 0.937.

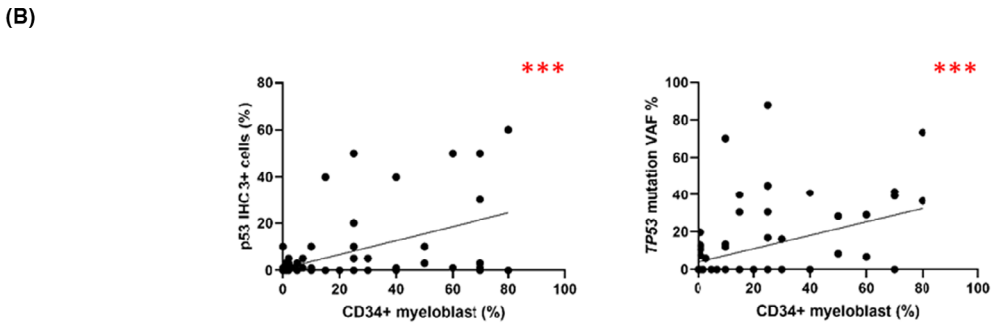
Among the 72 neoplastic cases, 25 showed ≥ 10% total p53 expression (34.72%), 32 cases (44.44%) showed ≥ 1% strong (3+) p53 expression, 17 cases (23.61%) showed ≥ 5% strong (3+) p53 expression and 12 cases (16.66%) showed ≥ 10% strong (3+) p53 expression. Regarding the mutation status, 26 of 72 cases (36.11%) carried at least one *TP53* mutation, with eight cases (11.11%) having multiple *TP53* mutations (Table 1). Six further cases (8.33%) harboured bi-allelic *TP53* alterations due to evidence of *TP53* copy loss. None of the reactive cases displayed strong (3+) p53 expression or carried *TP53* mutations. However, mild to moderate p53 expression was observed in three of the four reactive cases, with a percentage of 1–20% (Figure 2).

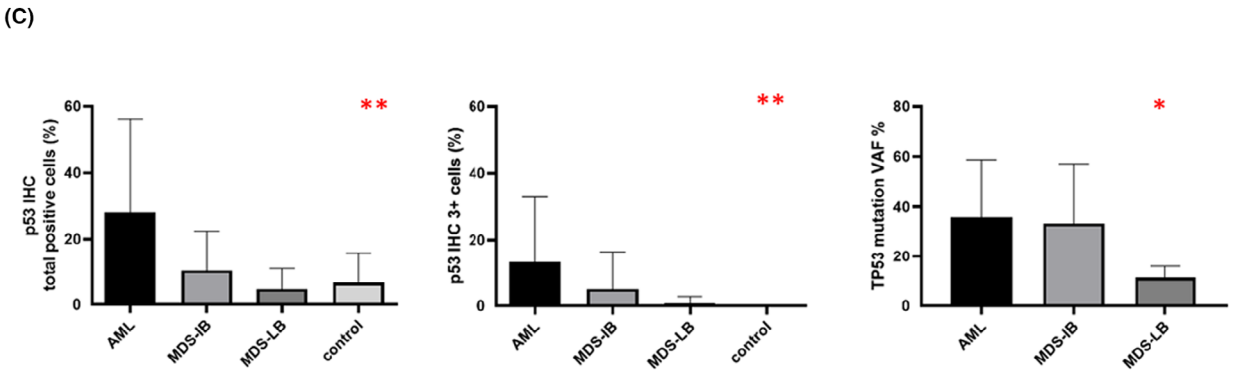
CORRELATION BETWEEN P53 EXPRESSION AND MYELOBLAST COUNT

Myeloblast count showed a significant correlation with both total p53 expression (Spearman's $r = 0.4470$, $P < 0.0001$) and strong (3+) p53 expression (Spearman's $r = 0.4880$, $P < 0.0001$; Figure 1B). Cases were categorised into three groups based on the myeloblast count: AML (≥ 20% blast count, n = 26), MDS-IB (5–19% blast count, n = 12), MDS-LB (< 5% blast count, n = 34). A significant difference was detected between these subgroups both in total and strong (3+) p53 expression ($P = 0.0039$ and $P = 0.0011$, respectively, as assessed by the Kruskal–Wallis test; Figure 1C).

We performed double IHC examinations on cases displaying ≥ 10% total p53 expression. Our observations revealed that p53 nuclear expression frequently occurs together with CD34 positivity, although not exclusively. On one hand, p53−/CD34+ cells could be observed while, conversely, the p53+/CD34− cell population was also identifiable. Despite statistical results indicating a significant correlation between the proportion of p53-positive bone marrow cells and the myeloblast ratio, based on the double IHC examinations, it could be concluded that p53 expression is not







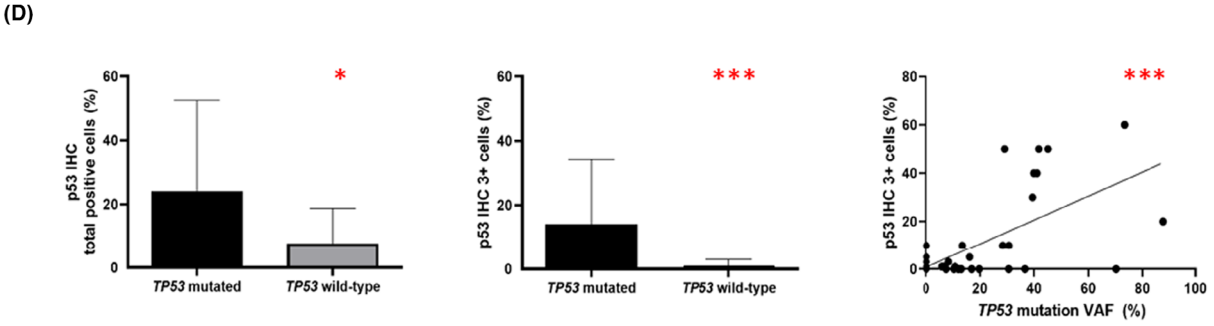


Figure 1. Correlation between *TP53* status, p53 expression and myeloblast count in bone marrow biopsy specimens with dysplasia-related alterations (n = 76). **A,** Heat-map summarising the distribution of cases according to myeloblast count [acute myeloid leukaemia, myelodysplastic neoplasm (AML, MDS-IB and MDS-LB)], *TP53* status, total and strong (3+) p53 expression, and comprehensive cytogenetic scoring system (CCSS). *TP53* mutated cases showed a variance accounted for (VAF) value of less than 10%, coverage below 500, frameshift mutations or having more than two mutations are highlighted, as these mutated cases were frequently associated with a low-level p53 expression. **B,** Correlation of strong (+) p53 expression and *TP53* VAF value with bone marrow myeloblast count. Both the percentage of p53 3+ cells and *TP53* mutation VAF value showed a significant correlation with the percentage of CD34+ myeloblasts (Spearman's r = 0.4880, $P < 0.0001$ and Spearman's r = 0.4757, $P < 0.0001$, respectively). **C,** P53 expression and *TP53* VAF value in different disease subgroups. A significant difference was detected between these subgroups both in total and strong (3+) p53 expression (P = 0.0039 and P = 0.0011, respectively, as assessed by the Kruskal–Wallis test). The median VAF values varied significantly between MDS-LB, MDS-IB and AML subgroups (Kruskal–Wallis test, P = 0.016). **D,** Correlation between p53 expression and *TP53* mutation status. The percentage of bone marrow cells with total or strong (3+) p53 expression was significantly higher in the *TP53* mutant group compared to the *TP53* wild-type group, as assessed using the Mann–Whitney test (P = 0.0417 and P = 0.0005, respectively). The VAF value showed a significant correlation with strong (3+) p53 expression ($P < 0.0001$). ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

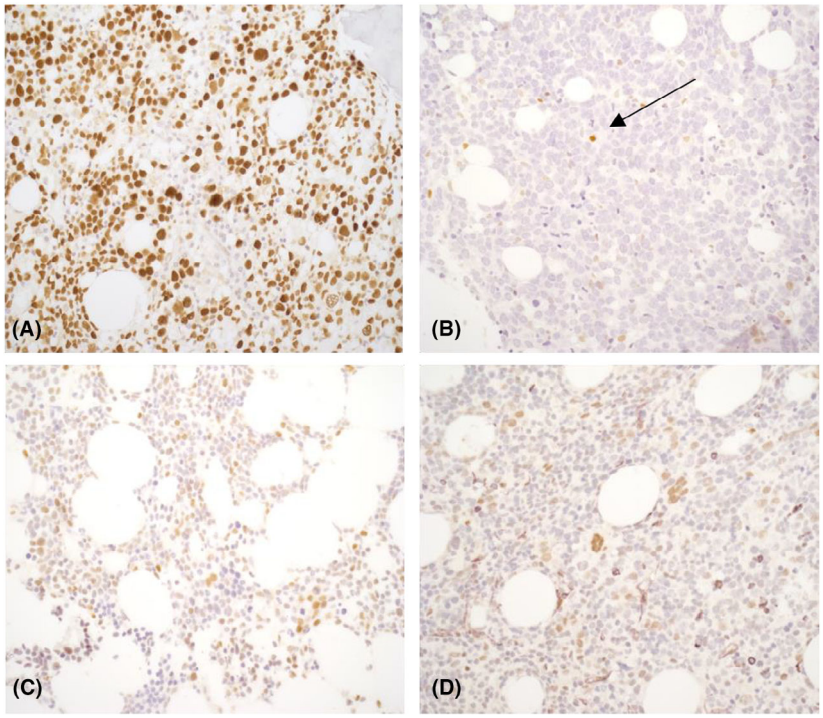


Figure 2. Typical p53 expression pattern of bone marrow biopsy samples: **A,** Acute myeloid leukaemia, myelodysplasia-related (AML-MR) case with two *TP53* mutations. The variance accounted for (VAF) value was higher than 10% for both mutations. More than 10% of bone marrow cells display strong (3+) p53 expression. **B,** AML-MR case harbouring one *TP53* mutation without the evidence of a bi-allelic state, VAF: 36.66%. The *TP53* alteration in this case is a frameshift mutation causing protein truncation and instability. Both total and strong (3+) p53 expressions were below 10%. A few isolated positive cells are detectable (arrow) and probably belong to the residual reactive haematopoiesis. **C,** AML-MR, *TP53* wild-type. Strong (3+) p53 expression is not present. Some scattered cells display weak to moderate p53 expression. **D,** Reactive case with transient cytopaenia, *TP53* wild-type. Isolated bone marrow cells show p53 expression with variable intensity.

exclusively associated with the myeloblast cell population. Notably, the p53 expression was often associated with the erythroid cell lineage, which was confirmed by the double staining of p53+/CD71+. Furthermore, in several cases, p53 expression was observed in cells exhibiting megakaryocyte morphology (Figure 3). The ratio of p53-positive cells ranged widely in all analysed cell lineages. The ratio of p53-positive erythroblasts ranged between 0 and 50%; this range was 0–75% for megakaryocytes and 1–95% for CD34+ myeloblasts.

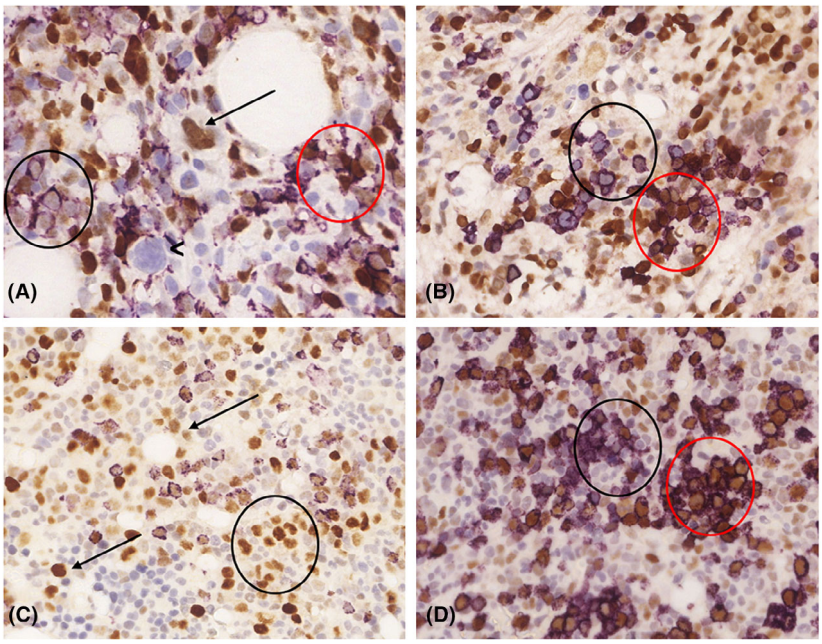


Figure 3. Double immunohistochemical stains of two cases with acute myeloid leukaemia (AML). Double immunohistochemical staining underscores the complexity of interpreting p53 immunohistochemistry. The p53 expression varies in intensity and can manifest across various cell lineages. Reactive, non-clonal and clonal cells are admixed, which adds further complexity to the evaluation. **A,B,** An AML case harbouring one *TP53* mutation with a 41.73% variance accounted for (VAF) value; the myeloblast ratio is 70%, the total p53 labelling is 80%. **A,** The p53/CD34 double immunohistochemical stain reveals three distinct patterns – cells with p53+/CD34− (scattered throughout the sample), p53+/CD34+ (red circle) and p53−/CD34+ (black circle) cells pattern could be observed. One p53-positive (arrow) and one p53-negative (arrowhead) dysplastic megakaryocyte are also present (p53: brown, DAB/CD34: purple, VIP). **B,** The p53/CD71 double immunohistochemical stain detects p53−/CD71+ (black circle) and p53+/CD71+ (red circle) erythroid cells. A p53+/CD71− population is also present, which probably corresponds to myeloblasts (original magnification: ×400; p53: brown, DAB/CD71: purple, VIP). **C,D,** An AML case harbouring one *TP53* mutation with a 16.20% VAF value; the myeloblast ratio is 30%, the total p53 labelling is 40%. **C,** The p53/CD34 immunohistochemical stain highlights numerous p53+/CD34− cells with variable p53 labelling intensity (arrows) (p53: brown, DAB/CD34: purple, VIP). Considering the relatively low VAF value, it is possible that weak p53 labelling is associated with non-mutated cells exhibiting physiological p53 expression. **D**, The p53/CD71 highlights p53−/CD71+ (black circle) as well as p53+/CD71+ (red circle) erythroid cells (p53: brown, DAB/ CD71: purple, VIP).

CORRELATION BETWEEN TP53 MUTATION STATUS AND MYELOBLAST COUNT

The ratio of *TP53* mutated cases proportionally reflected the myeloblast count as 20.59% (seven of 34) of MDS cases, 33.33% (four of 12) of MDS-IB cases and 57.69% (15 of 26) AML cases were mutated. The ratio of multiple TP53 mutations showed a similar tendency: 2.94% (one of 34) of MDS cases, 16.67% (two of 12) of MDS-IB cases and 19.23% (five of 26) of AML cases carried multiple mutations. The median of VAF values varied significantly between MDS-LB (VAF median = 10.81, mean = 11.39), MDS-IB (VAF median = 30.65, mean = 33.27) and AML (VAF median = 33.68, mean = 35.84) subgroups, as assessed by the Kruskal–Wallis test ($P = 0.016$). The VAF value was significantly higher in AML and MDS-IB compared with MDS-LB, as assessed with the Mann–Whitney test ($P = 0.0056$ and $P = 0.303$, respectively), while there was no significant difference between AML and MDS-IB ($P = 0.7539$; Figure 1C). These findings are consistent with the observation that the VAF value showed a significant correlation with the myeloblast count (Spearman's $r = 0.4757$, $P < 0.0001$; Figure 1B).

CORRELATION BETWEEN P53 EXPRESSION AND TP53 MUTATION STATUS

Of the 26 *TP53* mutated cases, 18 carried only one mutation, while eight had multiple mutations. The VAF value, which ranged from 5.04 to 88.04% with a mean of 22.75% and a median of 24.08%, exhibited a significant correlation with both total and strong (3+) p53 expression (Spearman's $r = 0.2953$,

$P = 0.0096$ and Spearman's $r = 0.4398$, $P < 0.0001$, respectively).

The percentage of bone marrow cells with strong (3+) p53 expression was significantly higher in the *TP53* mutant group compared to the *TP53* wild-type group, as assessed using the Mann–Whitney test (mean 14.15 versus 0.98%, respectively, $P = 0.0005$; Figure 1D).

A significant association was found between p53 expression and *TP53* mutation status using all the defined cut-off values. The highest specificity (0.9800) was observed with the ≥ 10% strong (3+) p53 cut-off value, indicating that 49 of the 50 wild-type cases were negative, but the sensitivity (0.4231) was low, with only 11 of the 26 mutant cases being positive. Conversely, the highest sensitivity (0.6154) was seen using the 1% strong (3+) p53 expression as a cut-off value, with 16 of the 26 mutant cases showing positivity, but the specificity (0.6800) was relatively lower, with 34 of the 50 wild-type cases being negative (Table 2).

Of the 26 *TP53* mutated cases, 14 (53.85%) were considered to harbour bi-allelic alteration. In eight cases multiple mutations were detected, in three cases the VAF value exceeded 50% and in three further cases one *TP53* mutation was present, together with chromosome 17 deletion. In 12 cases (46.15%), a single *TP53* mutation was detected without the evidence of a bi-allelic state. Four of 12 (33.33%) cases with mono-allelic *TP53* alteration and 64.29% (nine of 14) of cases with bi-allelic *TP53* alteration displayed ≥ 10% total p53 expression; this difference was not significant ($P = 0.2377$). There was no significant difference in the percentage of strong (+3) p53-positive cells between cases with mono-allelic and bi-allelic *TP53* alterations (mean = 8.83 versus 19.64%, respectively, Mann–Whitney test, $P = 0.0754$).

CHARACTERISTICS OF CASES WITH BI-ALLELIC TP53 ALTERATIONS

In three cases with a single *TP53* mutation (three of 18) the VAF value was > 50%, which suggests the loss of the second allele. Among these cases, two showed ≥ 10% strong (3+) p53 expression while one case was completely negative for p53. The negative case carried the *TP53* p.Y163Xfs mutation, leading to p53 protein truncation. Two of these three cases were AML-MR and one was MDS-*biTP53* with a ≥ 10% myeloblast count.

Eight further cases carried multiple *TP53* mutations (Table 3). Four of these cases (four of eight) showed ≥ 10% strong (3+) p53 expression. Further analysis revealed that in three of the four negative cases more than two mutations were present, resulting in potential p53 protein instability and consequent loss of expression. Moreover, two of these cases carried stop codon (p.C135X) or frameshift mutation (p.K373Rfs). Finally, in two cases the VAF values were relatively low (< 10%). These factors may explain the low-level p53 expression. Clinically, five cases were AML-MR, two cases were myeloid neoplasms post-cytotoxic therapy (MN-pCT) and one case was MDS with an increased blast count. This MDS case is classified as MDS-*biTP53*. All cases with ≥ 10% p53 expression had a > 10% myeloblast count.

Three further cases were considered to harbour bi-allelic *TP53* alteration due to the simultaneous presence of chromosome 17 deletion and *TP53* mutation. Two of these cases (two of three) showed ≥ 10% strong (3+) p53 expression, while one case was completely negative. The latter case carried a frameshift mutation (p.E286Qfs). Clinically, all these cases were classified as AML-MR with > 10% myeloblast count.

Table 2. Accuracy of strong (3+) and total p53 expression for the predicting *TP53* mutations. The highest specificity and positive predictive value were present with ≥ 10% strong (3+) p53 expression, while the highest sensitivity and negative predictive value were observed with a threshold of ≥ 1 strong (3+) p53 expression

	Strong (3+) p53 expression ≥ 1%	Strong (3+) p53 expression ≥5%	Strong (3+) p53 expression ≥ 10%	Total p53 expression ≥ 10%
Sensitivity	0.6154	0.4615	0.4231	0.5000
Specificity	0.6800	0.9000	0.9800	0.7600
Positive predictive value	0.5000	0.7059	0.9167	0.5200
Negative predictive value	0.7727	0.7627	0.7656	0.7451

CHARACTERISTICS OF TP53 MUTATED CASES WITHOUT THE EVIDENCE OF BI-ALLELIC STATE

In 12 cases only one *TP53* mutation was present, the VAF value was < 50% and evidence of a *TP53* locus deletion in 17p13 was not present. Four cases showed ≥10% strong (3+) and five cases ≥ 10% total p53 expression, while in seven cases a discrepancy was present, as both total and strong (3+) p53 expression were < 10%. In one AML-MR case a truncating mutation was detected (p.L93Lfs), and in three AML-MR cases the coverage was low (< 500) indicating a minor *TP53* mutant population in the sample. These circumstances may explain the negative IHC result. A further six *TP53* mutant MDS cases showed negative IHC, two of which carried *TP53* mutation with a relatively low VAF (5.94 and 7.52%). In four MDS-LB cases, however, no convincing explanation was found, as both VAF and coverage were satisfactory, and no truncating mutation was present. All cases with ≥ 10% p53 expression had a ≥ 10% myeloblast count.

INCONSISTENCY BETWEEN TP53 STATUS AND P53 EXPRESSION

Among the 26 *TP53* mutated cases, 15 were AML, four were MDS-IB and seven were MDS-LB. In 13 cases ≥ 10% total p53 expression was present (11 of 15 AML, two of four MDS-IB, none of seven MDS-LB). In 13 further cases, total p53 expression was < 10% (four of 15 AML, two of four MDS-IB, seven of seven MDS-LB). The discrepancy was the most prominent in the MDS-LB group, as none of the mutated cases showed ≥ 10% p53 expression. As described previously, satisfactory possible explanations were present in all the p53 low-expressor (< 10%) *TP53* mutated AML and MDS-IB cases. Frameshift mutations, multiple mutations with protein instability, low VAF value with a minor clone or low coverage were present in these cases. Conversely, in four MDS-LB cases there was no apparent mutation-related explanation found for this inconsistency; however, the myeloblast count was low. It was also observed that all cases with mutated *TP53* and ≥ 10% total p53 expression also had a myeloblast count greater than 10% (Figure 1A).

CORRELATION BETWEEN CCSS SCORE AND P53 STATUS

A comprehensive cytogenetic scoring system (CCSS) score was available in 60 neoplastic cases (Table 1). There was a significant difference in CCSS score between *TP53* mutated and wild-type cases ($P < 0.0001$, χ^2 test for trend). Complex karyotypes and a consequential very poor CCSS score were more frequent in *TP53* mutated cases. Very poor CCSS score was present in 52.38% (11 of 21) of *TP53* mutated cases and in only 7.14% (three of 42) of *TP53* wild-type cases. Altogether, very poor CCSS score was present in 14 cases, 11 of which were *TP53* mutated.

The CCSS score also showed significant differences between p53-negative (< 10%) and p53-positive (≥ 10%) cases ($P < 0.0001$, χ^2 test). Very poor CCSS characterised 87.5% (seven of eight) of p53-positive cases and only 12.73% (seven of 55) of p53-negative cases.

Discussion

Myelodysplastic neoplasm (MDS) represents a diverse group of clonal haematopoietic stem cell disorders. To ensure effective treatment strategies, optimising risk stratification for individual MDS patients is essential. *TP53* mutations have been established as adverse risk factors, associated with high cytogenetic complexity and monosomal karyotypes in both MDS and AML.[1]

The results of this retrospective study revealed a significant correlation between *TP53* mutation status and p53 protein expression. Various cut-off values for p53 expression were explored, and it was observed that a ≥ 10% strong (3+) p53 expression cut-off provided the highest specificity while a 1% strong (3+) p53 expression cut-off had the highest sensitivity. Moreover, the VAF value was found to be significantly correlated with both total and strong (3+) p53 expression. There was no significant difference in p53 expression between cases with mono-allelic and bi-allelic alterations.

Several authors have explored the relationship between *TP53* mutation status and p53 protein expression, observing a strong correlation.[24–26] P53 immunohistochemistry is directed to the initial, non-involved region of the protein by the commonly used Do-07 antibody clone. Increase in labelling is an indirect sign of mutations resulting mutant/wild-type protein dimerisation and delayed breakdown of the protein.[28–31] It has been published previously that p53 immunopositivity itself is associated with a poor prognosis. However, the precise cut-off value for p53 expression remains unclear. The distinction between bi-allelic and mono-allelic alterations concerning immunopositivity is also subject to question. Additionally, further investigation is needed to determine

Table 3. Characteristics of *TP53* mutated cases with multiple mutations (n = 8). Cases with more than two mutations were associated with a low level (< 10%) strong (3+) p53 expression (cases 4, 6 and 7). One additional case with a low VAF value (< 10%) also displayed a similarly low p53 expression (case 3). In contrast, the other four cases showed ≥ 10% strong (3+) p53 expression

Case	WHO diagnosis	*TP53* AA change	VAF (%)	Number of mutations	Total p53 expression	Strong (3+) p53 expression	Myeloblast count	Cytogenetics
1	AML-MR	p.E271K	27.8	2	80%	50%	60%	CK
		p.N239D	29.14					
2	AML-MR	p.V272M	30.7	2	20%	10%	25%	ND
		p.Y220X	8.38					
3	AML-MR	p.E271K	6.08	2	3%	1%	60%	CK
		p.N239D	6.67					
4	AML-MR	p.E271K	8.28	3	10%	3%	50%	Modal chromosome number: 78–83
		p.M246K	8.35					
		p.C135X	5.65					
5	AML-MR	p.V216M	39.44	2	70%	30%	70%	CK
		p.G245S	38.22					
6	MN-pCT	p.S215N	30.65	6	3%	0%	15%	ND
		p.K373Rfs	11.59					
		p.T256I	6.82					
		p.P98L	6.25					
		p.Q375E	5.6					
		p.T253I	5.45					
7	MN-pCT	p.S362N	5.19	4	1%	1%	1%	46, xy
		p.G334R	5.04					
		p.V272M	6.27					
		p.N239D	10.81					
8	MDS-biTP53	p.R248Q	39.93	2	40%	40%	15%	CK
		p.P152Q	38.21					

WHO, World Health Organisation; AML-MR, acute myeloid leukaemia myelodysplasia-related; MN-pCT, myeloid neoplasms post-cytotoxic therapy; VAF, variance accounted for; CK, complex karyotype; ND, not done.

the distribution of p53-positive cells among bone marrow haematopoietic cell lineages.

Interestingly, some cases exhibited *TP53* mutations without ≥ 10% p53 protein expression, and vice versa. This incongruence could be attributed to the presence of multiple *TP53* mutations, leading to protein instability and subsequent loss of p53 expression. Other contributing factors may include truncating mutations, low VAF value or low coverage during analysis. Protein instability and truncation lead to a completely negative staining pattern in solid tumours; however, this pattern is challenging to discern in bone marrow samples. Residual non-neoplastic bone marrow cells may express p53 with variable frequency. Based on our observations, the reactive cells with physiological p53 expression are intermixed with the neoplastic cells and prevent the entirely negative 'null' phenotype that is typical of solid tumours

with truncating mutations. A recently published large study examined the correlation between *TP53* mutation status and p53 immunostaining with digital image analysis.[32] Digital analysis provide a reliable, objective method to assess immunohistochemical stains and is especially useful in researches. In the absence of digital image analysis, < 5% and > 10% cut-off values of 3+ p53 expression were recommended by the authors for $p53^{wt}$ and $p53^{high}$ cases, while completely negative cases were defined as $p53^{tuncated}$. The authors stated that $p53^{truncated}$ pattern was easily recognisable; however, weak p53 expression of stromal cells were also observable in these cases and served as an internal control.

Notably, the most prominent discrepancy was observed within the MDS-LB group, suggesting that this subgroup may be particularly affected by the conflicting results. Furthermore, it was observed that all cases with mutated *TP53* and p53-positive immunostaining had a myeloblast count greater than 10%. This finding implies that p53 immunopositivity is not solely dependent upon the presence of *TP53* mutations, but also correlates with the myeloblast count in the analysed samples. Low myeloblast counts indicate the parallel occurrence of clonal and normal, non-clonal haemopoesis. Their actual balance may influence the particular *TP53*/p53 findings.

TP53 mutation and p53 expression showed a correlation with the myeloblast count. Specifically, the ratio of *TP53* mutated cases increased proportionally with the myeloblast count, with 20.59% of MDS cases, 33.33% of MDS with increased blast count (MDS-IB) cases and 57.69% of AML cases carrying *TP53* mutations. The VAF value was significantly higher in AML and MDS-IB cases compared to MDS cases, indicating a potential association between higher VAF and a higher percentage of myeloblasts. The myeloblast count showed a significant correlation with both total and strong (3+) p53 expression. Cases with higher blast counts were more likely to exhibit increased p53 expression.

Based on these observations, one would expect that p53 protein expression is exclusively present only in myeloblasts. However, double IHC stainings of samples with > 10% total p53 expression revealed that, in most cases, erythroid cells and megakaryocytes exhibit partial p53 expression. These cells showed a p53+/CD34− pattern. We should consider that at least a proportion of these lineages are part of the process and also derivate from the mutant stem cell clone. The ratio of positively stained erythroids ranged between 1 and 50%, while this range was 0–75% in megakaryocytes. The CD34+ myeloblasts exhibited only partial p53 expression (1–95%), as p53−/CD34+ cells were also present. The strong expression of neoplastic erythroid cells has already been described in the literature, mainly in erythroid leukaemia. A recent publication has shown that strong diffuse p53 expression maybe a useful additional marker in the differential diagnosis of erythroid leukaemia and reactive erythroid proliferations.[33]

Conclusions

The findings from this study contribute to a clearer understanding of the role of *TP53* mutations and p53 protein as an indicator of the mutant phenotype in MDS and AML. The findings suggest that with proper interpretation, p53 immunohistochemistry can be a valuable tool for the identification of cases with *TP53* mutations with excellent specificity but a low sensitivity. Immunohistochemistry could be especially useful in cases with increased blast counts. However, this tool is not sufficient for the distinction between mono-allelic and bi-allelic alterations, and molecular analysis including the karyotyping is essential. The observations underscore the complex relationship between *TP53* mutations and p53 protein load and highlight the need for further investigations to elucidate the underlying mechanisms.

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Conflicts of interest

The authors declare no conflicts of interest.

Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of University of Debrecen (IRB reference number: 60355-2/2016/EKU and IV/8465-3/2021/EKU).

Data availability statement

Data available on request from the authors.

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