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Immunohistochemical pattern of p53 is a measure of TP53 mutation burden and adverse clinical outcome in myelodysplastic syndromes and secondary acute myeloid leukemia

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Running head: P53 IHC correlates with mutant TP53, poor outcome

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Myelodysplastic syndromes (MDS) are genetically diverse malignancies with peripheral cytopenias, dysplastic hematopoiesis, and increased risk for acute myeloid leukemia (AML) transformation. Recent investigations indicate that somatic, myeloid-specific gene mutations refine clinical staging to alter estimates of overall survival (OS) and should be included in current risk stratification models.¹ Hence, identification of these mutations and corresponding protein expression levels has rising clinical utility. TP53 mutations are found in 5-10% of MDS patients, are enriched in patients with isolated del(5q), complex cytogenetics, or MDS with fibrosis (MDS-F), and are associated with overall worse prognosis.¹⁻⁵ Next-generation sequencing (NGS) is a valuable ancillary tool, however, the technology may not be economically feasible for routine community use. Alternatively, immunohistochemical (IHC) is fast, reproducible, and cost effective for routine laboratory use. In this study, we explore the relationship between p53 expression and TP53 gene mutation in MDS and acute myeloid leukemia with myelodysplasia-related changes (AML-MRC). Additionally, we investigate correlations between p53 expression and clinical characteristics including TP53 mutation variant allele frequency (VAF), myeloblast percentage, cytogenetic characteristics and outcome.

Patients diagnosed at Moffitt Cancer Center 7/2013-1/2015 with NGS (n=201) were retrospectively retrieved. Those informative for TP53 mutations diagnosed with MDS, MDS/ MPN, or secondary AML-MRC (proceeding from MDS or MDS/MPN) were included. TP53 mutant patients (13 MDS, 9 AML-MRC, mean age 67.3 years) with available bone marrow trephine biopsies (>1cm) at the time of sequencing (n=22) were compared to 32 patients without TP53 mutation [wild-type (WT) cases] (27 MDS, 5
AML-MRC, mean age 70.0 years) and 5 hematologically normal controls. Diagnosis was based on the 2008 World Health Organization criteria. P53 IHC was performed using standard protocol (p53 antibody, clone Bp53-11, Ventana Medical Systems, Tucson, AZ). Nuclear p53 expression was assessed quantitatively by percent p53 positivity and semi-quantitatively with an IHC score of stain intensity (0, 1+, 2+, and 3+) multiplied by percent positive hematopoietic cells. NGS was performed by Genoptix Inc. (Casbah, CA) using a gene panel including ASXL1, CBL, DNMT3A, ETV6, EZH2, IDH1, IDH2, JAK2, KIT, MPL, NPM1, NRAS, PHF6, RUNX1, SETBP1, SF3B1, SRSF2, TET2, TP53, U2AF1, ZRSR2. Student’s t or Chi-square tests were used to calculate significance (p<0.05). Correlations were analyzed using Pearson’s coefficient. Kaplan-Meier curves were constructed to estimate OS and compared by log rank.

The results of the study showed that AML-MRC diagnoses were significantly overrepresented in patients with mutant TP53 compared to WT cases (40.1% AML-MRC cases versus 15.5%, respectively, p=0.037). Similar to Saft et al. who studied lower-risk del(5q) MDS patients, we found significantly higher p53 IHC staining in mutant compared to WT TP53 patients [IHC score (mean ± SE), 1.11±0.16 versus 0.037±0.077, respectively; percent positivity 38.59%±5.30% versus 2.16%±0.919%, respectively]. Notably, only mutated TP53 cases had statistically significant increased staining compared to normal controls (Figure 1 A-B, p<0.001). Representative photomicrographs are provided in Figure 1C. The sensitivity and specificity of the p53 IHC score (1.0 cutoff) in predicting TP53 mutation status was 59.1% and 100% respectively, and 77.3% and 100%, respectively, when using a 0.5% percent positive cutoff.
Among all cases, we found a significant positive correlation between rising cytogenetic risk, defined according to IPSS or R-IPSS (we applied the risk classifications to both MDS and AML cases to increase cohort numbers), and p53 positivity or IHC score (p<0.001). There were statistically significant increases in p53 expression (by positivity and score) in mutant versus WT cases in IPSS low (p<0.05) or high (p<0.001) cytogenetic risk groups (Figure 2 A-B). Similar results were observed using the R-IPSS. Significance was not reached in intermediate patients due to low numbers. We next investigated the relationship between IHC score and p53 positivity to any 17p abnormality assessed by karyotyping and chromosome 17p and 5q deletions assessed by FISH. Indeed, we found a significantly greater p53 staining by p53 positivity and IHC score in patients with 17p abnormality (p=0.012 and p=0.015, respectively), 17p deletion (p=0.014 and p=0.010, respectively), or 5q deletion (p<0.001 and p<0.001, respectively) (Figure 2 C-H). We also found a significant increase in p53 IHC expression measured by percentage and score in those with complex cytogenetics (≥3 abnormalities) (p<0.001), and a significant positive correlation between the absolute number of cytogenetic abnormalities and p53 expression (r=0.689, p<0.001, positivity; r=0.677, p<0.001, score). In addition, we found a significant positive correlation between bone marrow blast percentage and p53 expression (r=0.362, p=0.007, positivity; r=0.313, p=0.021, score) (Figure 2 I-J). Recently, we demonstrated that prognostic significance of TP53 mutation is integrally related to VAF; hence, we also investigated the relationship between TP53 mutation VAF and p53 IHC. We found a striking association between TP53 VAF and p53 expression (r=0.867, p<0.001, positivity; r=0.8555, <0.001, score) (Figure 2 K-L) in patients with MDS. We were
unable to demonstrate a statistically significant correlation between TP53 VAF and p53 expression in AML-MRC case which could be attributed to small sample size as a positive trend was noted (r=0.346, p=0.147, positivity; r=0.345, p=0.148, score).

As expected, we found OS was significantly (p=0.001) diminished in patients harboring mutant TP53 compared to WT. Median OS was 15.0 months (CI 95%, 8.2-21.8) for mutated patients versus not reached in WT (Figure 3A). To discern a useful prognostic threshold of p53 positivity we used 2 cutoff points, 0.5% and 1.0%. Differences in OS reached statistical significance using both cutoffs in patients exceeding the threshold (p=0.070 and p=0.031, respectively) (Fig 3B). Median OS in cases with 0.5% or greater p53 positivity was 40 months (CI 95%, 14.1-65.9) and was not reached in cases with <0.5% positivity. Similar analyses were performed using the IHC score at 0.5 and 1.0 cutoffs. Using either, we found statistically significant (p=0.004 and 0.002, respectively) inferior OS in threshold exceeding cases (Figure 3C). Median OS for cases with an IHC score >0.5 was 15 months (CI 95%, 6.1-23.9) versus not reached in cases below. Using cox regression, we determined that both percent positivity and p53 IHC score predicted OS. The IHC score proved a more powerful predictor (HR=2.62, CI 95% 1.47-4.69) compared to percent positivity (HR=1.03, CI 95% 1.01-1.05).

In this investigation, we demonstrated that profound increases in cellular p53 expression are associated with TP53 mutation, higher risk disease, and inferior OS. This was expected as mutant p53 expression is often upregulated due to ineffective clearing by the E3-ubiquitin ligase and primary negative regulator, MDM2. Similar to recent findings in lower-risk, del(5q) MDS and MDS-F, we demonstrate significantly
increased p53 IHC expression in patients with a TP53 mutation in non-cytogenetically specific MDS and AML-MRC patients.\textsuperscript{7,9} To define a prognostically important cutoff, we used a range of scores based on the percent positivity as well as staining intensity and found a statistically significant decrease in OS in those patients with score >0.5 (p=0.004) or positivity >0.5\% (p=0.070). Although prognostically relevant in this data set, the threshold warrants validation in a larger cohort. In the Saft et al. report, a cutoff of 1\% p53 positive cells with 3+ signal intensity distinguished between low and high p53 expression.\textsuperscript{7} In our study, we created an IHC score taking into account both percentage of positive cells and signal intensity, and propose that this score has broader utility.

Similar to a recent study, we also found that increased p53 expression was significantly associated with 17p abnormalities and 17p and 5q deletions in the study.\textsuperscript{9} Furthermore, we also confirmed that there was significantly increased p53 expression in patients with complex cytogenetics and a positive correlation between increased p53 expression and blast count. Recently, we found that TP53 VAF is associated with inferior OS, and further refines prognosis over binary mutation analysis.\textsuperscript{5} Importantly, we found here, that p53 expression is positively and significantly associated with TP53 mutation VAF in MDS patients (p<0.001), a finding not previously reported. Collectively, these data provide more evidence for the clinical applicability of p53 IHC in assessment of prognosis and TP53 somatic gene mutation status.

Although NGS is becoming standard at diagnosis in academic centers, it is not be universally available. Alternatively, p53 IHC is a feasible alternative to TP53 sequencing. IHC is standardized, results are available faster than NGS and costs are 30-50x less suggesting IHC as a good alternative to facilitate management decisions.
Since \textit{TP53} mutations are one of the most powerful prognostic factors in MDS\textsuperscript{1,3}, it is imperative that clinicians have a quick, reliable surrogate tool to rapidly identify such mutations in settings where NGS is not readily available. Triaging patients via IHC results would potentially decrease health care costs while increasing usefulness of available diagnostic tools to identify driver mutations. As Bejar et al. suggested, the presence of molecular abnormalities such as somatic gene mutations should be used to further refine prognosis reclassifying patients into higher risk categories\textsuperscript{1}. Similarly, perhaps the quick and widespread availability of an assay to identify higher risk for such mutations, such as p53 IHC, could similarly be used to reclassify patients. As a potentially powerful risk stratifier, the data here should be validated in a large cohort to define specific cutoffs for inclusion in future prognostic scoring systems.

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References Cited

**Figure Legends**

**Figure 1.** Increased p53 IHC staining is associated with TP53 mutation. (A). Significantly higher p53 IHC staining as assessed by p53 percent positivity in mutated versus WT TP53 cases, both of which are greater than controls.  (B) Significantly higher p53 IHC staining as assessed by p53 IHC score in mutated versus WT cases, both of which are greater than controls. (C) (a-f) Representative micrographs of p53 signal intensity. (a) Negative for p53 (intensity=0, magnification x600) (b) weakly positive for p53 (intensity=1+, magnification x1000) (c) moderately positive for p53 (intensity=2+, magnification x1000) (d) strongly positive for p53 (intensity=3+, magnification x1000) (e) variably positive for P53 (intensity=1+ to 2+, magnification x1000) (f) representative semi-quantitative scoring (2.5% positive x 3+ intensity=7.5 intensity score, magnification x 600). (g-o) Representative micrographs of increased p53 expression in mutant TP53 patients. Bone marrow core biopsy from a patient with low grade MDS and WT TP53 (g. H&E, magnification x600; h. and i. P53 immunoperoxidase, magnification x200 and x600, respectively). (j-l) bone marrow core biopsy from a patient with RAEB MDS and mutant TP53 (j. H&E, magnification x600; k. and l. p53, immunoperoxidase, magnification x200 and x600, respectively). (m-o) bone marrow core biopsy from a patient with sAML-MRC with mutant TP53 (m. H&E, magnification x600; n. and o. p53, immunoperoxidase, magnification x200 and x600, respectively).

**Figure 2.** Increased p53 expression is associated with increased cytogenetic risk, chromosomal abnormalities, bone marrow blast count and TP53 VAF. Significant increase in p53 positivity (A) and IHC score (B) in patients with mutated TP53 by
cytogenetic risk group defined by IPSS. Increased p53 positivity in patients with 17p abnormalities assessed by karyotyping (C), or 17p deletions assessed by FISH (D), or 5q deletions assessed by FISH (E). Similar results were observed using the IHC score (F-H). Significant positive association between bone marrow blast percentage and percent positive p53 IHC staining (I) and p53 IHC score (J) as well as between TP53 VAF and percent positive p53 IHC staining (K) and p53 IHC score (L).

Figure 3. Kaplan Meier plots for OS by TP53 mutation and p53 IHC expression. (A) Significantly inferior OS in cases with mutated TP53 compared to WT TP53. (B) Inferior OS in patients with a low percent positivity of p53 staining using a 0.5% cutoff. (C) Significantly inferior OS in cases with a lower IHC score using a 0.5 cutoff.
Figure 2. Increased p53 IHC expression is associated with increased cytogenetic risk defined by IPSS

A. P53 Positivity by WT and Mutant
   - WT (n=32)
   - Mutant (n=22)
   - LOW INT HIGH
   - * **

B. IHC Score
   - WT (n=22)
   - Mutant (n=32)
   - LOW INT HIGH
   - * **

C. P53 positivity by 17p Abnormality
   - p=0.012
   - No 17p Abnorm (n=36)
   - 17p Abnorm (n=9)

D. P53 positivity by 17p Deletion
   - p=0.010
   - No 17p deletion (n=36)
   - 17p deletion (n=9)

E. P53 positivity by 5q Deletion
   - p<0.001
   - No 5q deletion (n=24)
   - 5q Deletion (n=21)

F. IHC Score by 17p Abnormality
   - p=0.015
   - No 17p Abnorm (n=36)
   - 17p Abnorm (n=9)

G. IHC Score by 17p Deletion
   - p=0.014
   - No 17p deletion (n=36)
   - 17p deletion (n=9)

H. IHC Score by 5q Deletion
   - p<0.001
   - No 5q deletion (n=24)
   - 5q Deletion (n=21)

I. r=0.362
   - P=0.007

J. r=0.313
   - P=0.021

K. r=0.867
   - P<0.001

L. r=0.855
   - P<0.001
Figure 3. Kaplan Meier plots for OS by TP53 mutation and p53 IHC expression

A. P53 Mutation
- No mutation
- P53 mutation
- Censored
- Censored

B. P53 Percent Positive
- Percent positive ≤ 0.5%
- Percent positive > 0.5%
- Censored
- Censored

C. IHC Score
- Percent positive ≤ 0.5
- Percent positive > 0.5
- Censored
- Censored