The relationship of TP53 R72P polymorphism to disease outcome and TP53 mutation in myelodysplastic syndromes

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discernible functional differences. The R/R homozygous genotype has greater apoptosis-promoting potential than the P/P genotype, arising in part from intrinsically greater mitochondrial localization triggering cytosolic release of cytochrome c.16,18 The homozygous P/P genotype displays greater transcriptional efficiency, inducing a higher level of G1 arrest than the R/R genotype in a tissue- and context-specific manner.16,19–23

Previous studies of the R72P variants in hematologic malignancies have shown conflicting results. Patients with chronic lymphocytic leukemia harboring a homozygous C allele displayed inferior clinical outcome irrespective of immunoglobulin heavy-chain variable (IGHV) gene mutation status,24 whereas no similar relationship was observed in patients with acute myeloid leukemia (AML).25 In chronic myelogenous leukemia, however, C-allele homozygosity was associated with decreased overall survival (OS) and progression-free survival (PFS).26 A meta-analysis of TP53 R72P association with hematologic malignancy risk validated an association of R72P with lymphoma risk, but not leukemia. Previous reports of R72P in MDS involved only small patient cohorts and did not examine the relationship to chromosome 5q deletion.27 Given the pathogenetic importance of p53 in del(5q) MDS, we investigated the distribution of this SNP in patients with MDS vs controls, and explored the relationship to TP53 mutations, chromosome 5q deletion, disease features and outcome.

MATERIALS AND METHODS

Study population, tissue specimens and DNA isolation Peripheral blood and/or bone marrow were collected from patients (n = 705, 372 non-del(5q) and 333 del(5q)) and healthy controls (n = 157) who provided written consent according to Institutional Review Board (IRB)-approved protocols. Controls were not biologically related to cases and were either Florida blood bank donors or recruited on Moffitt Cancer Center clinical trials with IRB consent. All controls were healthy individuals. MDS cases were collected from hospitals in the United States (Moffitt Cancer Center (DER and AFL), Cleveland Clinic (JPM) and Arizona Cancer Center (AFL)), Canada (British Columbia Cancer Center (AK)), Spain (Hospital de Mar, ICO-Hospital Germans Trias I Pujol, Hospital Universitario La Fe and Hospital Clinico Universitario Santiago de Compostela (FS)), Germany (MLL Munich Leukemia Laboratory (TH)) and London (King’s College Hospital (GMI)). DNA was isolated using Qiagen (Valencia, CA, USA) DNA isolation kits according to the manufacturer’s instructions. Median follow-up for all patients was 34.7 months for OS and 43.2 months for PFS.

PCR and TPS3 genotyping DNA was amplified using GoTaq Green (Promega Cooperation, Madison, WI, USA) according to the manufacturer’s protocol. Up to 100ng of DNA was used to amplify TPS3 exon 4 using primers F-5′-CCTGGCTCTGGAGTCGC TCTTTTCACCA-3′ and R-5′-GGCCAGCGTGAAGGCTCGT-3′ under the following conditions: 94°C for 2 min, 35 cycles of 94°C for 30 s, 58°C for 30 s and then 72°C for 30 s, followed by a final 5 min extension at 72°C, and held at 4°C. PCR products were resolved on a 2% agarose gel, and the exon 4 356 bp fragment was excised and purified using Wizard SV Gel and PCR Cleanup Kit (Promega Cooperation). The purified DNA was sequenced by the Sanger method using BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and a 3130xl Genetic Analyzer (Applied Biosystems). Genotypes were identified by sequencing in both forward and reverse directions. German samples were sequenced by next-generation deep sequencing of the exons 4–11 of the TPS3 gene using the 454 GS FLX amplicon chemistry (Roche Applied Science, Indianapolis, IN, USA) as described previously.28 For London samples, 14 amplicons across the entire TPS3 coding region, including untranslated exon 1, were sequenced using the Roche GS FLX sequencing platform (Roche, Indianapolis, IN, USA) as described previously.29

Variable description Clinical data on all patients was collected through March 2011, and disease-specific features analyzed were limited to the date of diagnosis. Genotype frequency did not differ across races among del(5q) MDS cases (P = 0.54) or controls (P = 1.0); however, there was a significant difference in TP53 genotype between Caucasian and non-Caucasian among non-del(5q) MDS informative cases, or in those of which these data were available (P = 0.05). As the proportion of non-Caucasian patients within each subgroup was <10% (3.6% for del(5q), 10% for non-del(5q) and 3.6% for controls), and we found no significant difference in R72P genotype by race (P = 0.21) in the total population, all analyses included all patients and controls regardless of race. Cytogenetic risk was defined according to the following criteria: low/good: normal, –Y and isolated 5q or 20q deletion as single anomaly; intermediate (Int): 5q deletion plus one, or one abnormality; and high/poor: any chromosome 7 abnormality or 2+ abnormalities. R72P genotype among World Health Organization (WHO) subtypes was assessed by comparing genotype distribution across the following categories: isolated del(5q), refractory anemia with excess blasts-1 (RAEB-1), RAEB-2, RAEB-transformed (RAEB-T) and ‘other’, which included refractory anemia (RA), RA with ring sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD) and RCMD with ring sideroblasts; MDS/myeloproliferative neoplasm (MPN), AML and the non-proliferative MDS/MPN chronic myelomonocytic leukemia (CMML).

Statistical analysis Participants’ demographic features, TP53 genotype and allele frequency among controls and MDS (non-del(5q) and del(5q)) cases were compared using the analysis of variance test for continuous variables and the χ2 test using the exact method with Monte Carlo estimation for categorical variables. The association between R72P genotype and disease features was evaluated using the Fisher’s exact test. OS was defined as the interval from the date of diagnosis until the date of death from any cause or until the last date of follow-up. PFS was defined as the time from diagnosis to development of AML or to date of death due to any cause if the patient did not ever develop AML. When patients developed de novo AML, the time of transformation to AML was censored and the time to death from AML was recorded. The Kaplan–Meier graph by genotype was generated and compared using the log-rank test. The genotype frequencies between del(5q) MDS patients with deletions resolved by metaphase cytogenetics were analyzed using the χ2 test using the exact method with Monte Carlo estimation. All P-values ≤ 0.05 were considered statistically significant. All analyses were conducted using SAS (Version 9.2; SAS Institute Inc., Cary, NC, USA).

RESULTS

Subject characteristics A total of 705 MDS cases were analyzed (372 non-del(5q) and 333 del(5q)), as well as 157 healthy controls. The mean age in controls was 53.3 years and ranged from 18 to 89 years (Table 1). Among informative non-del(5q) MDS, the mean age was 68.5 years with a range of 16–94 years, and for del(5q) patients mean age was 68.7 years and ranged from 23 to 93 years. The percentage of females in each population was 52.8%, 32.2% and 66.5% in controls, non-del(5q) and del(5q) MDS, respectively, as isolated 5q– is more frequent in females; however, there were no genotype differences by sex. WHO MDS subtype was available for 348 non-del(5q) cases, which included 50 (14.4%) refractory anemia with excess blasts-1 (RAEB-1), 32 (9.2%) RAEB-2, 4 (1.1%) AML, 10 (2.9%) non-proliferative MDS/MPN category of CMML, 4 (1.1%) MDS/MPN-unclassifiable and 24 (7.1%) ‘other’. The non-blastic, morphologic subtypes of refractory anemia (RA), RARS, RCMD and RCMD with ring sideroblasts. Within the del(5q) cohort, WHO subtype was available for 309 cases, which included 179 (57.9%) patients with isolated del(5q) MDS, 28 (9.1%) with RAEB-1, 17 (5.5%) with RAEB-2, 5 (1.6%) with RAEB-T, 15 (4.9%) with AML, 2 (0.6%) with CMML and 63 (20.4%) with ‘other’ diagnoses (Table 1). Of the 321 non-del(5q) cases with International Prognostic Scoring System (IPSS) data, 254 (79.1%) were grouped as lower risk, which included both low-risk and intermediate-1 IPSS values, whereas 67 (20.9%) were grouped as higher risk, which included intermediate-2 and high-risk patients. Of the 164 del(5q) cases informative for IPSS, 72% were lower-risk and 28% were higher-risk patients by IPSS. Distribution of low, intermediate and high cytogenetic risk
among 357 non-del(5q) patients was 265 (74.2%), 52 (14.6%) and 40 (11.2%), respectively, and 213 (68.9%), 21 (6.8%) and 75 (24.3%), respectively, among 309 del(5q) cases.

R72P genotype and disease status
To assess potential differences in allele distribution and frequency, we first compared SNP genotype among controls and cases of non-del(5q) and del(5q) MDS and found no significant differences in R72P genotype ($p = 0.13$), including no significant difference between the del(5q) vs non-del(5q) cases ($p = 0.17$). The frequency of CC, CG and GG among controls, non-del(5q) and del(5q) samples were 7.6%, 37.6% and 54.8%; 9.7%, 44.6% and 45.7%; and 12.6%, 38.1% and 49.2%, respectively (Table 1). There was no significant difference in allele frequency in del(5q) MDS vs non-del(5q) MDS cases ($p = 0.91$); however, differences between non-del (5q) allele frequency and controls approached significance ($p = 0.08$), as well as del(5q) cases, compared with controls ($p = 0.10$). C-allele frequency for controls, non-del(5q) MDS and del(5q) MDS was 26.4%, 32.0% and 31.7%, respectively (Table 1). These findings suggest that there may be greater C-allele frequency among MDS cases compared with controls.

### Table 1. Case–control comparison of R72P genotype

| Control | Non-del(5q) | Del(5q) |
|---------|-------------|---------|
| Total, n | 157         | 372     | 333    |
| Age at sampling, (mean, range) | 53.3, 18–89 | 68.5, 16–94 | 68.7, 23–93 |
| Percent female, n | 52.8 (85) | 32.2 (120) | 66.5 (216) |
| Percent Caucasian, n | 95.9 (94) | 90 (325) | 96.2 (187) |
| WHO classification, n | 348 | 309 | 3 | 39 |
| Isolated 5q | 0 (0) | 57.9 (179) | 14.4 (50) |
| RAEB-1 | 14.4 (50) | 9.1 (28) |
| RAEB-2 | 9.2 (32) | 5.5 (17) |
| RAEB-T | 0 (0) | 1.6 (5) |
| MDS/MPN | 1.1 (4) | 0 (0) |
| CMML | 2.9 (10) | 0.6 (2) |
| AML | 1.1 (4) | 4.9 (15) |
| Other | 71.3 (248) | 20.4 (63) |
| CC genotype (%), n | 7.6 (12) | 9.7 (36) | 12.6 (42) |
| CG genotype (%), n | 37.6 (59) | 44.6 (166) | 38.1 (127) |
| GG genotype (%), n | 54.8 (86) | 45.7 (170) | 49.2 (164) |
| C-allele frequency (%) | 26.43 | 31.99 | 31.68 |
| G-allele frequency (%) | 73.57 | 68.32 | 68.01 |

### Table 2. R72P genotype frequency by clinical characteristics

| | Non-del (5q) | Del (5q) |
|---|-------------|---------|
| | CC | CG | GG | CC | CG | GG |
| IPSS grouping (%) | 10 (32) | 44.2 (142) | 45.85 (147) | 15.9 (28) | 44.5 (73) | 39.6 (65) |
| Low | 9.4 (24) | 46.1 (117) | 44.5 (113) | 16.9 (20) | 48.3 (57) | 34.7 (41) |
| High | 11.9 (8) | 37.3 (25) | 50.7 (34) | 13 (8) | 34.8 (16) | 52.2 (24) |
| P-value | 0.421 | 0.019 | 0.119 |
| Cytogenetics risk group (%) | 9.5 (34) | 45.4 (162) | 45.1 (161) | 12.3 (38) | 37.2 (115) | 50.5 (156) |
| Iso-del(5q) or NA | 9.8 (26) | 45.7 (121) | 44.5 (118) | 10.8 (23) | 37.6 (80) | 51.6 (110) |
| Del(5q) T+1 Ab | 9.6 (5) | 44.2 (23) | 46.2 (24) | 9.5 (2) | 52.4 (11) | 38.1 (8) |
| Complex | 7.5 (3) | 45 (18) | 47.5 (19) | 17.3 (13) | 32 (24) | 50.7 (38) |
| P-value | 0.992 | 0.019 | 0.325 |
| WHO classification | 9.8 (34) | 45.5 (158) | 44.7 (155) | 12.6 (39) | 38.2 (118) | 49.2 (152) |
| Isolated del(5q) | 0 (0) | 0 (0) | 0 (0) | 8.9 (16) | 36.3 (65) | 54.7 (98) |
| RAEB-1 | 4 (2) | 54 (27) | 42 (21) | 17.9 (5) | 39.3 (11) | 42.9 (12) |
| RAEB-2 | 18.6 (6) | 28.1 (9) | 53.1 (17) | 11.8 (2) | 52.9 (9) | 35.3 (6) |
| RAEB-T | 0 (0) | 0 (0) | 0 (0) | 20 (1) | 40 (2) | 40 (2) |
| RA, RARS, RCMD, RCMD-RS | 9.3 (23) | 46.6 (115) | 44.1 (109) | 17.5 (11) | 39.7 (25) | 42.9 (27) |
| CMML | 30 (3) | 50 (5) | 20 (2) | 0 (0) | 0 (0) | 100 (2) |
| AML | 0 (0) | 100 (4) | 26.7 (4) | 40 (6) | 33.3 (5) | 0.041 |

% Abbreviations: AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; RA, refractory anemia; RAEB-1, refractory anemia with excess blasts-1; RARS, RA with ring sideroblasts; WHO, World Health Organization. *Non-proliferative MDS/MPN.  

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and GG, respectively) \( (P = 0.08\) and \( P = 0.69, \) respectively). Age at diagnosis did decrease with G-allele homozygozity in non-del(5q) MDS and approached significance when comparing the age at diagnosis in patients harboring the GG genotype between non-del(5q) and del(5q) MDS patients \( (P = 0.07).\)

R72P genotype and disease natural history
To investigate possible differences in outcome by R72P genotype, we assessed OS and PFS. There was no significant difference in OS by genotype among all MDS cases (log-rank test, \( P = 0.64\)), where 5-year OS was 0.46, 0.56 and 0.49 for CC, CG and GG, respectively (Figure 1). Among non-del(5q) cases, median survival by genotype was 4.5 years for CC, 10.2 years for CG and 5.3 years for GG \( (P = 0.39).\) However, 1-year survival increased in a G-allele dosage manner with survival estimates of 0.83, 0.96 and 0.98 for CC, CG and GG. Among del(5q) MDS patients, the median OS was 4.9 years for CC, 5.1 years for CG and 4.0 years for GG \( (P = 0.52)\) with >1-year survival associated with the C-allele \( (0.82, 0.77\) and 0.73 for CC, CG and GG, respectively). Although there was no significant differences in PFS in del(5q) MDS by R72P genotype \( (P = 0.53)\) or non-del(5q) MDS \( (P = 0.61),\) the CC genotype showed a trend of overall improved PFS than G-allele-containing genotypes in del(5q) MDS, whereas G-allele-containing genotypes were associated with improved PFS and OS in non-del(5q) MDS (Figure 2). When comparing the interaction of the R72P genotype with other prognostic variables (i.e., age at diagnosis, cytogenetics, blast percentage and gender) by multivariate analysis, only IPSS retained prognostic significance for OS and PFS.

R72P genotype and lenalidomide response duration
We previously reported that lenalidomide modifies p53 stability, which facilitates G1 escape and transition to G2 arrest in del(5q) progenitors.\(^{30,31}\) To determine if TP53 R72P SNP genotype influences lenalidomide response or duration, we explored the relationship between R72P genotype and duration of erythroid response to lenalidomide treatment in del(5q) patients. Erythroid response was defined using the previously described criteria.\(^{1,2,32}\) Although we found no significant difference in response rates by genotype \( (P = 0.75)\) (73.5% overall response rate, 71.4% for CC, 77.5% for CG and 69.0% for GG), comparison of response duration by genotype showed a trend for increased duration of transfusion independence with C-allele dosage; however, this did not reach statistical significance \( (P = 0.51)\) (Figure 3). Median response durations according to genotype were 2.2, 1.3 and 0.89 years for CC, CG and GG genotypes, respectively. Interestingly, the 2-year estimate for lenalidomide response duration was more than doubled for CC \( (0.50)\) compared with both CG \( (0.21)\) and GG \( (0.13)\) \( (P = 0.09).\)

Chromosome 5q deletion size, TP53 mutation and R72P genotype
Size of the deletion on chromosome 5q varies from microdeletions to large terminal deletions. The larger, more terminal lesions, particularly those including or extending telomeric to 5q34, have been linked to higher-risk MDS and AML, and unfavorable prognosis. To ascertain the relationship between R72P genotype and extended terminal chromosome deletions, we investigated genotype distribution by deletion inclusion of chromosome 5q34 deletion (Figure 4a). Using a cohort of 190 del(5q) patient

Figure 1. Kaplan–Meier estimates of OS according to R72P genotype. Non-del(5q) MDS (left) and del(5q) MDS (right).

Figure 2. Kaplan–Meier estimates of PFS according to R72P genotype. Non-del(5q) MDS (left) and del(5q) MDS (right).

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specimens, chromosome 5 deletion location was assessed by diagnostic metaphase cytogenetics. We found that the GG genotype was significantly associated with those patients harboring a deletion involving 5q34 ($P = 0.05$) compared with those patients with a C-allele-containing genotype. The frequency of a 5q34 deletion in either CC or CG samples was 50%; however, this increased to 71% for those with a GG genotype. Jerez et al.\textsuperscript{33} recently reported an association between larger chromosome 5 deletions and TP53 mutations;\textsuperscript{33} therefore, we next examined the relationship between R72P genotype and TP53 DNA-binding domain mutation. Among the 211 del(5q) cases with TP53 mutation analysis, 30.3% contained a DNA-binding domain mutation; however, there was no association with R72P genotype (IC, 45.5, CG, 23.9, and GG, 31.4%). Among those patients harboring the 5q34 deletion, we found no association with TP53 mutation ($P = 0.33$) (Figure 4b). Lastly, we found no association between GG genotype and TP53 mutation in the del(5q) cases ($P = 0.38$) (Figure 4c). There was no association with TP53 mutation and any R72P genotype in either del(5q) MDS ($P = 0.15$) or non-del (5q) MDS ($P = 1.0$).

**DISCUSSION**

Gene deletion studies indicate that the p53 polyproline domain is essential to mount an effective apoptotic response to stress and inhibit tumorigenesis.\textsuperscript{34–37} P53-P72 binds more weakly than p53-R72 to the positive regulatory protein PIN1, a prolyl isomerase,\textsuperscript{38} yet interacts more readily with the inhibitory protein iASPP (inhibitor of ASPP).\textsuperscript{39} We found no differences in age of MDS onset by R72P genotype; however, germline gene mutations implicated in the development of familial MDS, such as the Runx-related transcription factor-1 (RUNX1), similarly show no consistent association with earlier onset of disease, suggesting that secondary events necessary for MDS development are age-dependent.\textsuperscript{40–42}

A significant finding in this study was the association of the G-allele and GG homozygous genotype with more terminal 5q34 deletions ($P = 0.05$). Previous studies reported that patients with larger terminal deletions have a less favorable prognosis and higher frequency of TP53 mutation.\textsuperscript{33} Although we did not discern a relationship to gene mutation status, the overall frequency of TP53 gene mutation was relatively low. Nonetheless, the precise mechanisms driving the differences in deletion loci by R72P genotype remain unclear. The P72 (CC) variant is reported to be more efficient in inducing cell-cycle arrest and DNA repair than the R72 variant, which may account for the longer OS and PFS in del(5q) patients harboring the C-allele or CC genotype.\textsuperscript{23,43} By contrast, in patients with non-del(5q) MDS, we found that OS and PFS were reduced in those individuals harboring the CC genotype. We hypothesize that in non-del(5q) MDS in which the hematologic phenotype is not p53-dependent, the attenuated apoptotic response to stress and inhibited tumorigenesis may account for the reduced OS and PFS.

![Figure 3](image3.png) **Figure 3.** Kaplan–Meier estimate of lenalidomide (LEN) response duration in del(5q) MDS patients.

![Figure 4a](image4a.png) **Figure 4a.** Involvement of Chromosome 5q34 Deletion by R72P Genotype ($p = 0.05$).

![Figure 4b](image4b.png) **Figure 4b.** Chromosome 5q34 Deletion and TP53 mutation ($p = 0.33$).

![Figure 4c](image4c.png) **Figure 4c.** TP53 Mutation by R72P Genotype ($p = 0.38$).
activity of the C-allele/P72 variant may facilitate emergence and survival of clones with greater AML potential.

Although we were unable to discern significant differences in the duration of transfusion independence to lenalidomide according to genotype in del(5q) MDS patients, there was a trend favoring longer response duration in patients with a C allele that appears allele dosage dependent. As lenalidomide suppresses del(5q) MDS clones by inhibiting the dual specificity phosphatases CDCK2C5 and PP2Ac encoded within or adjacent to the 5q31 commonly deleted region to arrest progenitors in G0/M, we questioned whether the diminished apoptotic capacity of the homozygous CC phenotype might foster transition from G0/G1 and sustained G2/M arrest to account for the more prolonged response duration. Furthermore, given that approximately half of lenalidomide-treated patients become resistant to treatment within 3 years, the lack of significant differences in 5-year response duration is not surprising.1

These data underscore the distribution of R72P in MDS and highlight differences between del(5q) and non-del(5q) subtypes by gene polymorphism and the relationship to lenalidomide response. The potential interaction of R72P variants with germline variants in other key regulators or effectors of the p53 pathway further investigation in other large cohorts of patients.

CONFLICT OF INTEREST
AFL was a consultant for, and DB is employed by, Celgene Corporation. There are no other conflicts of interest to declare.

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AUTHOR CONTRIBUTIONS
KLM designed and performed research, collected and analyzed data, and wrote the manuscript; LMZ performed research and collected data; DER, AJ, WF, YZ, and generous support provided by Martin Schaffel.

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