Downregulation of p21 in Myelodysplastic Syndrome Is Associated With p73 Promoter Hypermethylation and Indicates Poor Prognosis

Youshan Zhao, MD, Juan Guo, MD, Xi Zhang, MD, Zheng Zhang, MD, Shucheng Gu, MD, Chengmin Fei, MD, Xiao Li, MD, PhD, and Chunkang Chang, MD, PhD

From the Department of Hematology, The Sixth Hospital affiliated to Shanghai Jiaotong University, Shanghai, China.

Key Words: p21; p73; MDS; Cell cycle

ABSTRACT

Objectives: p21 Can both promote and inhibit tumorigenic processes. We explored the role of p21 in myelodysplastic syndrome (MDS).

Methods: In this study, we analyzed p21 expression and p73 methylation in 88 patients with de novo MDS.

Results: We found decreased expression of the p21 gene in higher-risk MDS compared with lower-risk groups or healthy controls (P < .05). Patients with p73 methylation had lower p21 than those in the unmethylated group (P < .001). Moreover, there was a significantly positive correlation between p73 and p21 expression in MDS (r = 0.436, P < .001). In vitro assays further confirm the role of p73 methylation in p21 expression. Compared with patients with normal expression levels of p21, patients with lower p21 expression levels experienced much higher rates of transformation to acute myeloid leukemia and lower overall survival both in univariate as well as multivariate analyses.

Conclusions: Our results suggest p21 expression may serve as a new biomarker to predict clinical outcome in patients with MDS.

Myelodysplastic syndrome (MDS) is a heterogeneous group of clonal diseases derived from hematopoietic stem cells, which are characterized by ineffective bone marrow hematopoiesis, peripheral blood cytopenias, and substantial risk for progression to acute myeloid leukemia (AML). A review of the data supports the finding that in the early phase of the disease, increased apoptosis is associated with ineffective survival of progenitor and maturing hematopoietic cells, whereas in advanced stages of MDS, marrow cells have less apoptosis and exhibit more proliferative features. So, altered cell proliferation and apoptosis participate in progression of MDS. Abnormality of cell apoptosis is intensively investigated in MDS, but little is known about the changes of cell cycle and cell cycle–related molecules.

p21, A cyclin-dependent kinase inhibitor, is important in the response of cells to genotoxic stress. Originally, p21...
was considered to be a tumor suppressor. Decreased p21 expression has been observed in many solid tumors, such as colorectal, cervical, and small-cell lung cancers, and usually associated with poor survival. In hematopoietic malignancy, defective p21 activation has been implicated in leukemogenesis and an absence of p21 expression has been noted in T-cell acute lymphoblastic leukemia (T-ALL) cells. Our previous study found that p73, a gene with significant homology to p53, is able to induce p21 through Sp1/Sp3 sites. Moreover, in AML cells, induction of p21 expression is independent of its promoter methylation status but mediated by 5-Aza-CdR-induced re-expression of the tumor-suppressor p73. Our previous study found that in patients with MDS the p73 gene is frequently silenced by methylation of its promoter. Therefore, in the current study, we aimed to investigate whether p21 expression and cell cycle are abnormal in patients with MDS, and if so, whether p21 transcription is related to its p73 promoter methylation status. Moreover, we further explored the relationships between p21 expression and clinical outcomes in patients with MDS.

Materials and Methods

Patients and Samples

A total of 88 MDS cases were included in this study. The bone marrow (BM) samples were obtained at diagnosis before initiation of any treatment between 2009 and 2011 following approval by the local ethics committee and according to institutional guidelines. MDS was diagnosed in accordance with the minimum diagnostic criteria established by the Conference on MDS. The classification and prognostic risk scoring of MDS were performed according to the World Health Organization (WHO) criteria and the International Prognostic Scoring System (IPSS). Eighteen healthy volunteers were used as controls (median age, 51 years; range, 31-75 years), and they volunteered BM samples for this study. The health status of control subjects was monitored by an expert hematologist during the 6-month period before enrollment in the study. The main clinical and laboratory features of the patient cohorts are summarized in Table 1.

RNA Isolation and Real-Time Polymerase Chain Reaction (PCR) Analysis

Total RNA extraction from 1 mL of BM mononuclear cells (BMNCs) was performed with RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. The cDNA was prepared using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturer’s protocol. The primer sequences used for PCR were those described previously. Real-time PCR was performed in a final reaction volume of 10 μL containing 5 μL of RealMasterMix (Takara, Dalian, China), 0.5 μmol/L of each primer, and 1 μL of cDNA. The PCR was performed in a fluorescent quantitation PCR cycler (LightCycler, Roche, Switzerland). The amplification consisted of 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 20 seconds. The reactions were performed with an appropriate negative control in which the cDNA was replaced with diethylpyrocarbonate-treated water. The specificity of each primer pair was confirmed by melting curve analysis. The threshold cycle (Ct) value was calculated on the basis of the minimum diagnostic criteria established by the Conference on MDS. The classification and prognostic risk scoring of MDS were performed according to the World Health Organization (WHO) criteria and the International Prognostic Scoring System (IPSS). Eighteen healthy volunteers were used as controls (median age, 51 years; range, 31-75 years), and they volunteered BM samples for this study. The health status of control subjects was monitored by an expert hematologist during the 6-month period before enrollment in the study. The main clinical and laboratory features of the patient cohorts are summarized in Table 1.

Table 1

Demographic Data on 88 Patients With MDS

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. (%) of Patients</th>
<th>Mean ± SD p21 Expression</th>
<th>No. (%) of Cases With p73 Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>38 (43.2)</td>
<td>1.22 ± 0.89</td>
<td>15 (30.0)</td>
</tr>
<tr>
<td>≥60</td>
<td>50 (56.8)</td>
<td>1.07 ± 0.93</td>
<td>12 (31.6)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>53 (60.2)</td>
<td>1.07 ± 0.91</td>
<td>17 (32.1)</td>
</tr>
<tr>
<td>F</td>
<td>35 (39.8)</td>
<td>1.29 ± 0.91</td>
<td>10 (28.6)</td>
</tr>
<tr>
<td>WHO classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA/MDS-U/RARS</td>
<td>12 (13.2)</td>
<td>1.88 ± 0.94</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>RCMD/RCMD-RS</td>
<td>56 (62.5)</td>
<td>1.24 ± 0.89</td>
<td>12 (21.8)</td>
</tr>
<tr>
<td>RAEB-1/RAEB-2</td>
<td>21 (23.9)</td>
<td>0.54 ± 0.47</td>
<td>11 (52.4)</td>
</tr>
<tr>
<td>BM blast, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>67 (76.1)</td>
<td>1.33 ± 0.93</td>
<td>16 (23.9)</td>
</tr>
<tr>
<td>5–10</td>
<td>13 (14.8)</td>
<td>0.61 ± 0.52</td>
<td>6 (46.2)</td>
</tr>
<tr>
<td>≥11</td>
<td>8 (9.1)</td>
<td>0.48 ± 0.35</td>
<td>5 (62.5)</td>
</tr>
<tr>
<td>IPSS karyotypea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>59 (67.0)</td>
<td>1.30 ± 0.91</td>
<td>14 (23.7)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>12 (13.6)</td>
<td>1.06 ± 0.82</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>Poor</td>
<td>17 (19.3)</td>
<td>0.68 ± 0.82</td>
<td>9 (52.9)</td>
</tr>
<tr>
<td>IPSS risk groupsb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>10 (11.4)</td>
<td>1.94 ± 1.08</td>
<td>2 (20.0)</td>
</tr>
<tr>
<td>INT-1</td>
<td>52 (59.1)</td>
<td>1.33 ± 0.84</td>
<td>11 (21.2)</td>
</tr>
<tr>
<td>INT-2</td>
<td>22 (25.0)</td>
<td>0.52 ± 0.53</td>
<td>11 (50.0)</td>
</tr>
<tr>
<td>High</td>
<td>4 (4.5)</td>
<td>0.45 ± 0.33</td>
<td>3 (75.0)</td>
</tr>
</tbody>
</table>

BM, bone marrow; INT-1, intermediate 1; INT-2, intermediate 2; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome; MDS-U, MDS unclassifiable; RA, refractory anemia; RAEB, refractory anemia with excess blasts; RCMD, refractory cytopenia with multilineage dysplasia; RCMD-RS, RCMD with ringed sideroblasts.

a IPSS karyotype: good indicates normal, 5q− sole, 20q− sole, −Y sole; intermediate, not good or poor; poor, complex aberration or chromosome 7 abnormalities.

b IPSS risk groups: low indicates 0; INT-1, 0.5−1.0; INT-2, 1.5−2.0; and high, 2.5 or more, according to IPSS score.
expression level of normal controls), was expressed as $2^{-\Delta\Delta C_t}$ (fold difference), where $\Delta C_t = C_t$ of the target gene $- C_t$ of the endogenous control gene ($GAPDH$), and $\Delta\Delta C_t = \Delta C_t$ of the samples for the target gene $- \Delta C_t$ of the calibrator for the target gene.

**DNA Isolation and Bisulfite Modification**

BM samples from patients were harvested, and BMNCs were isolated using a Ficoll solution (Lymphoprep, Oslo, Norway) and then washed twice with phosphate-buffered saline. DNA was isolated from BMNCs using Genomic DNA Purification Kit (Tiangen, Beijing, China) following the manufacturer’s instructions. One microgram of genomic DNA was modified as described in the manufacturer’s instructions using the EZ-DNA methylation kit (Zymo Research, Los Angeles, CA). Modified DNA was resuspended in water and used immediately or stored at 80ºC until used.

**Methylation-Specific PCR (MS-PCR)**

The methylation status of exon 1 of $p73$ was determined with MS-PCR using the bisulfite-modified DNA. The MS-PCR was performed with primer pairs for the methylated $p73$ gene ($p73$-MF, 5’-GGACGTAGCGAAATCGGGGTTC-3’ and $p73$-MR, 5’-ACCCCGAACATCGACGTCG-3’) and for the unmethylated $p73$ gene ($p73$-UF, 5’-AGGGGATG-TAGTGAAATTGGGGTTT-3’ and $p73$-UR, 5’-ACCCACAACATCAACATCCA-3’) as described in our previous report.16 PCR was performed under the following cycling conditions: 95ºC for 5 minutes; 40 cycles of 95ºC for 30 seconds; specific annealing at 58ºC for 30 seconds; and 72ºC for 30 seconds; and a final extension of 10 minutes at 72ºC. The TaKaRa TaqTMHot Start Version was used in this study. PCR was performed in a PTC-200 cycler (Bio-Rad, Hercules, CA). The amplification products were analyzed on 2% agarose gels and visualized under UV illumination. The MS-PCR experiments were repeated 3 times.

**Western Blot Analysis**

$p21$ Protein expression in BMNCs from patients and healthy controls was evaluated with the Western blot. The cells were harvested and then washed once with cold phosphate-buffered saline. Whole-cell lysates were prepared with radio immunoprecipitation assay buffer containing 50 mmol/L Tris (pH 7.4), 1% NP40, 150 mmol/L sodium chloride, 1 m MEGTA, and 0.25% sodium deoxycholate in the presence of the protease inhibitors aprotinin (1 µg/mL), leupeptin (1 mmol/L), sodium vanadate (1 mmol/L), sodium fluoride (1 mmol/L), and phenylmethylsulfonyl fluoride (1 mmol/L). After normalization for total protein content (50 µg/lane), samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted using rabbit polyclonal antibodies anti-p21(Santa Cruz Biotechnology, Heidelberg, Germany) and anti-GAPDH (Huatesheng Biotechnology, Fushun, China), followed by horseradish peroxidase–conjugated donkey anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, England). Bound secondary antibodies were detected via chemiluminescence.

**Cell Cycle Analysis**

Cell cycle analysis was performed using ethanol-fixed cells stained with propidium iodide in buffer containing RNase A. DNA content was assessed with flow cytometry (Becton-Dickinson, Sunnyvale, CA), and cell cycle analysis was performed using the MULTICYCLE software package (Phoenix Flow Systems, San Diego, CA).

**Apoptosis Analysis**

The number of apoptotic cells was quantified using the Annexin-V-FITC Apoptosis Detection Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Early apoptotic cells were defined as Annexin-V-positive, 7AAD-negative cells. The analyses were performed on an FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA). The experiments were repeated 3 times.

**Cell Culture and Drug Treatment**

Decitabine was purchased from Sigma Chemical (St Louis, MO), and dissolved in phosphate-buffered saline at the appropriate concentrations. For the gene re-expression and demethylation studies, fresh BMNCs from patients with MDS or normal controls were treated with a final concentration of 1 µmol/L decitabine, harvested, and then subjected to RNA and DNA extraction, and analyzed to determine the level of cell cycle and cell apoptosis daily for 3 days. The drugs were added every day. The cells were plated at a density of $1 \times 10^6$ cells/mL in RPMI1640 medium (Gibco BRL, NY) containing 10% FCS, 2 mmol/L glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin in a 6-well tissue plate (Becton-Dickinson).

**Statistical Analysis**

All statistical analyses were performed with SPSS version 17.0 software (SPSS IBM, Chicago, IL). Two independent sample populations were compared using the Student $t$ test. Multiple sample populations were compared using one-way analyses of variance. The Pearson $\chi^2$ test was applied to compare the enumeration data between groups. The Pearson correlation analysis was used to examine the correlations between 2 parameters. The survival analysis was performed using the log-rank test and the Kaplan-Meier plot approach. The Cox regression model was used for the multivariate survival analysis. $P < 0.05$ was considered statistically significant.
Results

Patient Characteristics

A total of 88 patients with MDS, including 53 men and 35 women, were involved in this study. Their median age was 58 years (range, 19-84 years). Patients were classified as having MDS with refractory anemia (RA; n = 4), RA with ringed sideroblasts (RARS; n = 6), refractory cytopenia with multilineage dysplasia (RCMD; n = 52), RCMD with ringed sideroblasts (RCMD-RS; n = 3), refractory anemia with excess blasts 1 (RAEB-1; n = 13), RAEB-2 (n = 8), MDS with isolated del(5q) (n = 1), MDS unclassifiable (MDS-U; n = 1). Stratified by the IPSS, 10 patients were in low, 52 in intermediate 1 (INT-1), 22 in INT-2, and 4 in high-risk groups.

p21 Expression Levels and Cell Cycle Phase Distribution of BMNCs in Patients With MDS

The expression level of p21 \(2^{-\Delta\Delta CT}\) in normal controls was 1.31 ± 0.91 (mean ± SD). The p21 expression levels for patients with various subtypes of MDS were as follows: patients with MDS-U/RA/RARS/5q, 1.88 ± 0.94; patients with RCMD/RCMD-RS, 1.24 ± 0.89; patients with RAEB-1, 0.59 ± 0.53; and patients with RAEB-2, 0.44 ± 0.34. As shown in Figure 1, p21 expression was much lower in the WHO-classified RAEB-1 or RAEB-2 subgroup than in the RA/RARS/ MDS-U or RCMD/RCMD-RS subgroup or normal controls, and in the IPSS-classified INT-2/high-risk subgroup than in low/INT-1 subgroup \((P < .05\) for all subgroups). According to karyotype, patients with abnormal karyotype showed lower p21 than those with normal karyotype \((P < .05)\).

To further assess p21 expression, Western blot analysis was performed. Consistent with the PCR results, the expression of p21 protein in patients with higher-risk MDS (IPSS score \(\geq 1.5\)) was lower than in those with lower-risk MDS (IPSS score \(\leq 1.0\)) or healthy controls (Figure 1C).

The cell-cycle phase distribution of BMNCs was measured. The median value of S phase cells for patients with higher-risk MDS (IPSS score \(\geq 1.5\)) was statistically higher than in those with lower-risk MDS (IPSS score \(\leq 1.0\)) and healthy donors (5.66%) \((P = .019, .023\), respectively).

Figure 1. p21 mRNA and protein expression level in patients with myelodysplastic syndrome (MDS). A, Based on the World Health Organization classification, advanced MDS showed lower p21 expression levels than early MDS and normal controls. B, Patients with higher-risk disease (International Prognostic Scoring System [IPSS] scores \(>1.0\)) demonstrated lower p21 expression levels compared with those with lower-risk MDS. C, p21 Protein expression in patients with MDS.
Transcriptional Levels of p21 and p73 in Patients With MDS Are Associated With p73 Gene Methylation Status

As a known upstream of the p21 gene, p73 is often downregulated by promoter CpG hypermethylation in hematopoietic malignancy. p73-Dependent induction of p21 has been reported in many cell lines. Therefore the methylation status and the transcriptional levels of p73 were studied. As shown in our previous study, p73 promoter methylation was examined with MS-PCR. U937 cells were used as positive controls. As an MSP-positive band, all of the CpGs were methylated by bisulfite sequencing. Hypermethylation of p73 occurred in 27 patients (31%) but did not occur in any of the normal controls. The expression levels of p73 in normal BMNCs and MDS were 1.35 ± 0.73 and 0.91 ± 0.86, respectively. As shown in Figure 2, we found a positive correlation between p73 and p21 expression in MDS (r = 0.436, P < .001). According to the methylation status, the expression level of p73 in MDS with methylation (0.44 ± 0.35) was significantly lower than those without methylation (1.43 ± 0.87) (P < .001). In addition, p21 expression in patients with MDS with p73 methylation was lower than in those without methylation (P < .001).

Decitabine Treatment Restored p73 and p21 Expression and Induced Cell Cycle Arrest at the G2/M Phase

To confirm the role of p73 methylation status in p21 expression, demethylation experiments were carried out. The p73 methylation status and the gene expression levels were evaluated in 6 primary MDS (with methylation) samples and 6 healthy control samples both before and after treatment with 1 µmol/L of decitabine. After 3 days of decitabine treatment, MDS samples showed a demethylation effect, and the expression levels of p73 and p21 were increased (Figure 3). Although cell apoptosis was not changed, decitabine treatment increased the amount of G2/M phase cells from 0.47% (day 0) to 1.79% (day 3). However, the induction effect of decitabine on p21 and p73 expression was not found in BMNCs from normal controls harboring an unmethylated p73 promoter. In summary, the decitabine treatment restored the p73 and p21 expression and induced cell cycle arrest at the G2/M phase.

![Figure 2](image-url) The relationships between p73 methylation and gene expression. A, The expression of p73 mRNA in the group of patients with methylated samples was lower than in patients with unmethylated p73. B, Patients with p73 methylation demonstrated lower p21 expression levels compared with those with p73 nonmethylation. C, In patients with MDS, p21 expression was significantly positively correlated with p73 levels.
Lower Expression of \( \text{p21} \) Predicted Disease Progression and Poor Survival

We used a cutoff value of 1.0, with samples having \( 2^{-\Delta\Delta\text{Ct}} \) values less than 1.0 considered positive for lower expression. Lower \( \text{p21} \) expression was observed in 44 (50%) of 88 patients, which was more common in higher-risk patients with MDS than that in lower-risk patients (23/26 vs 21/62, \( P < .001 \)). During a median follow-up period of 26 months (range, 3-46 months), 55% (24/44) of patients with lower \( \text{p21} \) expression progressed to AML, compared with 18% (8/44) of patients with normal \( \text{p21} \) expression levels (\( \chi^2 = 12.57, P < .001 \)). Moreover, the overall survival (OS) rates of patients with lower \( \text{p21} \) expression were significantly lower than those of patients with normal \( \text{p21} \) expression levels (41% vs 80%, \( \chi^2 = 13.7, P < .001 \)).

To further evaluate the prognostic significance of \( \text{p21} \) expression, we performed both univariate and multivariate analysis. The results are presented in Table 2. In the univariate analysis, \( \text{p21} \) expression level, WHO classification (2001), marrow blast level, IPSS score, and IPSS karyotype had a significant impact on both OS and leukemia-free survival (LFS). Patients with lower \( \text{p21} \) expression progressed rapidly to AML (\( P < .001 \)) and had shorter survival (\( P < .001 \)) than those with normal \( \text{p21} \) expression. The median OS was 24 months for patients with lower \( \text{p21} \) expression compared with 38 months for patients with normal \( \text{p21} \) expression. We performed the multivariate Cox regression analysis of factors on OS or LFS, respectively. Multivariate analysis identified \( \text{p21} \) expression level and WHO classification as significant independent risk factors for poor OS time of patients with MDS. WHO classification had the strongest impact on OS (hazard ratio [HR] = 4.05). Moreover, both marrow blast levels and \( \text{p21} \) expression level showed a significant predictive value on LFS. Patients with MDS with lower \( \text{p21} \) expression had higher risk of leukemic transformation (\( P = .035, \text{HR} = 2.58 \)).

Discussion

The current study showed that low \( \text{p21} \) expression at the mRNA level is common in higher-risk patients with MDS and is associated with \( \text{p73} \) promoter methylation status. Lower expression of \( \text{p21} \) is an independent poor prognostic factor for OS in patients with MDS irrespective of karyotype, BM blast,
© American Society for Clinical Pathology

DOI: 10.1309/AJCPZ5E6IWPWSZXE

Figure 4 The overall survival (A; P < .001) and leukemia-free survival (B; P < .001) of patients grouped according to p21 expression level.

and IPSS score. In addition, patients having lower p21 expression would have a higher chance of disease transformation to AML. To the best of our knowledge, this is the first report to demonstrate the prognostic implication of p21 in MDS.

Deregulation of apoptosis and cell cycle are involved in the pathogenesis of MDS. Increasingly, p21 has been known as both a classic tumor suppressor and an oncogene, depending on the cellular context.24 An absence of p21 expression has been noted in T-ALL cells,9 whereas high levels of p21 in AML cells were associated with a poor prognosis.25 In this study, the p21 expression levels for patients with various subsets of MDS were measured. We found that p21 expression was much lower in the higher-risk group than in the lower-risk group or healthy controls, based on the WHO classification or IPSS score. These results are consistent with those of Economidou et al,26 who examined a series of mRNA expression levels of cell cycle and apoptosis regulatory genes in MDS and demonstrated that the expression of p21 was lower in patients with MDS than in healthy donors. We found that patients with MDS with abnormal karyotype showed lower p21 than did those with normal karyotype. It is well-known that a prominent feature of early-low-grade MDS is excessive apoptosis, whereas advanced MDS is characterized by apoptosis resistance and malignant clone proliferating. p21 is an inhibitor of cell cycle, and its lower expression in higher-risk MDS may favor clone proliferating and disease progression. In fact, the median value of S phase cells in higher-risk MDS was higher than that of normal individuals in our study.

We also investigated alternative mechanisms of dysregulated expression of the p21 genes. The p21 mutations are rare in human malignancies.27 Aberrant promoter CpG methylation has been shown to be involved in gene silencing, being equivalent to loss of function mutations or genetic deletion in MDS. However, absence of p21 methylation has been reported in MDS.28 Relying on the work of the pioneer Karin Schmelz, p21 is proved to be induced by demethylation of p73 in AML cell lines.13 Recently, we discovered that p73 methylation was common in patients with MDS. Our current study linked p21 expression with p73 methylation. As expected, patients with p73 methylation expressed less p21 than patients with p73 nonmethylation status, which suggests that p73 methylation may disrupt normal p21 expression.

Moreover, a positive correlation was found between p21 and p73 transcription. In vitro decitabine treatment restored p73 and p21 expression and induced cell cycle arrest at the G2/M phase. These experiments further confirmed the role of p73 gene methylation status in p21 expression in MDS. Further studies that include chromatin-immunoprecipitation and p73 knockdown by siRNA could provide a direct answer for the exact role of p73 demethylation in p21 induction.

p21 Can mediate cell cycle arrest in different phases. After DNA damage, p21 causes cell cycle arrest in the G1 phase,29 but it also plays a key role in the arrest in the G2/M transition.30 In this report, the increase in G2/M phase after decitabine demethylation in MDS cells is consistent with previous observations in other hematopoietic tumor cell lines.31,32 Of note, low expression of p21 is not only found in patients with p73 methylation, suggesting other mechanisms may be involved. According to reports, other epigenetic processes, such as histone deacetylase and microRNA, could likewise play a role in transcriptional inactivation of p21 in multiple cell lines.33-36

More importantly, we observed that downregulation of p21 expression was associated with disease aggressiveness.
Compared with patients with normal expression levels of p21, patients with lower p21 expression levels experienced much higher rates of transformation to AML and lower OS both in univariate as well as multivariate analyses. In our multivariate analysis, WHO classification and BM blast level, respectively, proved to have the strongest impact on OS (r = 4.05, P < .001) and LFS (r = 2.63, P < .001). These findings were consistent with previous large sample reports.\(^{19,27,38}\)

Previous studies suggested that very low levels of p21 protein expression was seen in most patients with AML,\(^{25,39}\) and high levels of p21 in AML cells were associated with a poor prognosis.\(^{22}\) Steinman et al\(^{40}\) reported that p21 prevents downmodulation of the apoptotic inhibitor protein c-IAP1 and inhibits leukemic apoptosis. In addition, a study indicated that p21 has an antiapoptotic role in acute monocytic leukemia when the protein is localized in the cytoplasm.\(^{41}\) These findings demonstrate that p21 may act as an oncogene in AML. However, in another study, p21 was detected in 5 (23%) of 22 cases of AML in immature myeloid cells, and not detected in immature myeloid cells in MDS.\(^{22}\) Therefore, the role of p21 in MDS may be different from its role in AML. It is also worth noting that the WHO classification, IPSS, and BM blast count are not truly independent because the WHO classification and IPSS incorporate blast count as part of the criteria. Therefore, the prognostic significance of p21 in MDS may be weak in correlation-type statements. Additional analyses in larger patient cohorts are required to confirm our observation.

To summarize, our study demonstrated that the expression of p21, which may be associated with p73 methylation status, was lower in higher-risk than in lower-risk subtypes of MDS. The BM p21 expression was an independent prognostic factor for LFS and OS in patients with MDS. It may serve as a new biomarker for foreseeing the clinical outcome and to stratify patients with MDS for risk-adapted treatment.

This study was supported in part by the National Natural Science Foundation of China (NNSFC81170463).

Address reprint requests to Dr Chang: Dept of Hematology, Shanghai Sixth People’s Hospital affiliated to Shanghai Jiaotong University, 200233 Shanghai, China; changchunkang7010@aliyun.com.

References