

# **Expression of CDKN1C in the bone marrow of patients with myelodysplastic syndrome and secondary acute myeloid leukemia is associated with poor survival after conventional chemotherapy**

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**Short title:** Impact of CDKN1C on outcome in MDS and AML

**Word count:** 4600

**Keywords:** CDKN1C, p57, survival, chemotherapy, MDS, AML

**Article type:** Research Article, Tumor Markers and Signatures

**NOVELTY AND IMPACT:** Our study shows that CDKN1C expression is an independent predictor of shorter survival in patients with MDS and secondary AML, particularly in those treated with conventional antiproliferative chemotherapy. CDKN1C may constitute a novel prognostic biomarker and may aid selection of patients most likely to benefit from aggressive treatment regimens. Our results may supplement current prognostic/predictive tools in order to facilitate and improve risk-adapted therapy of MDS and secondary AML.

## ABSTRACT

We tested the hypothesis, that proliferative activity of hematopoietic stem cells has impact on survival in newly diagnosed patients with myelodysplastic syndrome (MDS) and secondary acute myeloid leukemia (AML). RNA expression profiles of CD34<sup>+</sup> cells were analyzed in 125 MDS patients and compared to healthy controls. Prognostic impact on overall survival (OS) of mRNA proliferation signatures established for solid tumor cells was analyzed retrospectively. For validation on the protein level, immunofluorescence and immunohistochemistry analyses in bone marrow (BM) biopsies were performed, and an independent cohort of 223 MDS and secondary AML patients was investigated. Lower proliferative activity correlated with the expression of cyclin dependent kinase inhibitor 1C (*CDKN1C*) and with shorter OS ( $P<0.001$ ). In multivariable analysis, higher *CDKN1C* expression was associated with worse OS ( $P=0.02$ ). On the BM level, a total of 84 (38%) patients showed CDKN1C protein expression prior to start of treatment. Patient, disease and treatment characteristics did not differ between CDKN1C positive and negative patients. Positive CDKN1C BM status was associated with shorter OS in multivariable analysis (HR 1.54,  $P=0.04$ ). There was an interaction between CDKN1C BM status and subsequent treatment with negative impact on OS being most pronounced in patients receiving conventional cytotoxic chemotherapy (n=83, 2-year OS 30% *versus* 58%,  $P=0.002$ ). In conclusion, low-proliferative phenotype and *CDKN1C* expression were associated with shorter OS. CDKN1C protein expression in the BM of newly diagnosed, treatment-naïve MDS and secondary AML patients was identified as a prognostic factor for poor survival in patients treated with antiproliferative chemotherapy.

## INTRODUCTION

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal stem cell diseases associated with partial or complete bone marrow (BM) failure. There is a variable rate of transformation into (secondary) acute myeloid leukemia (AML) [1]. However, MDS is more than a pre-leukemic condition as only 20-40% of patients with excess of blasts develop AML whereas the majority of patients suffer and die from causes intrinsic to the disease (i.e. BM failure and the resulting cytopenias) [1-3]. Chemotherapy is largely inefficient in MDS. Although blast reduction is commonly achieved, most patients relapse or fail to recover their hematopoiesis [4]. For MDS, several classification and grading systems have been developed focusing on cytogenetics, cytopenias, and blast counts [3,5]. Furthermore, age and transfusion dependence also appear to predict outcome [6]. The transition to AML is defined by 20% blasts in blood and BM, however, this arbitrary cut-off poorly reflects the non-acute biology of the disease. In particular patients with a blast count of about 20-30% (termed oligoblastic AML) often progress slowly, very much unlike AML that has then to be formally diagnosed. For patients with AML, age and cytogenetics are the most relevant prognostic factors [7]. In addition, AML arising secondary to an antecedent hematologic disorder, and failure to achieve early blast clearance after first-line induction treatment remain poor prognostic factors even after early salvage [7,8]. In secondary AML patients, poor cytogenetics and a longer time span between onset of MDS and leukemic transformation was shown to negatively affect treatment outcome, so that a protracted history of MDS may select for higher rates of chemotherapy resistance when AML is treated [9]. Only 20-30% of patients with high-risk MDS and AML achieve long-term disease-free survival when treated with conventional chemotherapy [10,11]. For elderly patients, survival after chemotherapy is particularly poor with less than 10% being alive after 5 years [10]. Resistance to therapy remains the major cause of treatment failure and advanced age appears to confer resistance to

treatment [12]. This is an interesting point, since some older adults with AML may have a slower progressive clinical course adopting disease features of MDS [11].

The current cancer stem cell paradigm suggests that leukemia stem cells (LSC) are organized in a hierarchical order with primitive LSC giving rise to progeny with differing capacity for cellular differentiation and self-renewal [13]. LSC are characterized by cell cycle quiescence or dormancy. However, conventional chemotherapy is believed to predominantly target rapidly dividing cells [14]. Consequently, LSC may be prone to escape antiproliferative chemotherapy paving the way for disease recurrence [15,16]. In the present study, we tested the hypothesis, that lower proliferative activity caused by altered proliferation signatures within the stem cell compartment of the BM have prognostic impact on survival in treatment-naïve patients diagnosed with MDS and secondary AML.

## **PATIENTS, MATERIALS AND METHODS**

### **Patients recruited for gene expression profiling and immunohistochemistry analysis**

Gene expression profiling data on BM CD34<sup>+</sup> cells were obtained from a previous study [17]. This dataset contained data on 125 patients diagnosed with MDS according to WHO criteria [18] with a minimum follow-up of one year and 17 healthy controls (GEO Series accession number GSE19429). For retrospective immunohistochemistry analysis, a total of 223 patients with newly diagnosed MDS and secondary AML for whom trephine biopsies prior to start of treatment were available from two referral centers (Heidelberg and Düsseldorf) were included. In addition, 10 trephine biopsies obtained from patients with lymphoma who did not have BM involvement were used as control. Patient data were obtained from medical records and chart review. Cytogenetic risk was assessed according to published criteria [3,19]. Ethics committee's approval in all centers and informed consent for all patients was obtained according to the Declaration of Helsinki.

### **Gene expression profiling**

Gene expression profiling was performed as previously described [17]. The platform used is the GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA), covering over 47,000 transcripts representing 39,000 human genes. Gene expression data were preprocessed using GC-RMA, which is a modified version of the robust multiarray average (RMA) algorithm that describes the intensity of probes as a function of the GC-content [20]. Resulting gene expression levels were in log base 2 scale. To select a single representative probe set for each gene the method described in Li et al. [21] was applied using scoring methods to assess each probe set for specificity, splice isoform coverage, and robustness against transcript degradation. The overall score was then used to select the representative probe set for a gene to create a one-to-one mapping between genes and probe sets.

### **Immunofluorescence staining**

Immunofluorescence Ki67 and CD34 double staining was performed in 24 representative MDS patients (BM blast count <5%: n=6; BM blast count 5-10%: n=5; and BM blast count 11-20%: n=13) and 10 controls. After heat-induced epitope retrieval of paraffin-embedded tissue sections, samples were stained for Ki67 with a primary mouse monoclonal antibody (MIB-1, Dako, Copenhagen, Denmark) and a secondary anti-mouse fluorescein-isothiocyanate labeled antibody. Then, CD34 staining was done with a CD34 antibody (clone QBend10, 1:25, Dako, Copenhagen, Denmark) which was labeled with Alexa Fluor®555 (Zenon® Alexa Fluor® 555 Mouse IgG<sub>1</sub> Labeling Kit, Invitrogen, Germany). Images were acquired with a fluorescence microscope (Axioplan 2 imaging, Carl Zeiss, Jena, Germany). Because of the low number of CD34<sup>+</sup> cells in control trephine biopsies, double staining was performed by EnVision™ DuoFLEX Doublestain System (Dako). A total of 1000 CD34<sup>+</sup> were counted for each stained biopsy.

### **Real-Time RT-PCR quantification**

Separated fresh mononuclear cells of three patients with treatment-naïve MDS were labelled using anti-CD45-APC, anti-CD34-FITC and anti-CD38-PE antibodies (all Becton Dickinson, Heidelberg, Germany). Four subpopulations were sorted by FACS (CD34<sup>+</sup>CD38<sup>high</sup>, CD34<sup>+</sup>CD38<sup>low</sup>, CD34<sup>-</sup>CD38<sup>high</sup> and CD34<sup>-</sup>CD38<sup>low</sup>) and 50,000 cells were used for mRNA extraction (MagnaPure mRNA Isolation Kit I, Roche Applied Science, Mannheim, Germany). RT-PCR was performed using AMV-RT and oligo-(dT) as primer (First Strand cDNA synthesis kit, Roche Applied Science) according to the manufacturer's protocol. Primer sets specific for the sequences of CDKN1C and optimized for the LightCycler (RAS) were developed and provided by SEARCH-LC GmbH, Heidelberg. The PCR was performed with the LightCycler FastStart DNA SYBR GreenI kit (RAS) according to the protocols provided.

To correct for differences in the content of mRNA, the calculated copy numbers were normalized to the expression of the housekeeping gene peptidylprolylisomerase B (PPIB). Values were given as number of CDKN1C transcripts per 1000 PPIB transcripts.

### **Western Blot analyses**

CD34<sup>+</sup> positive cells were isolated from fresh BM aspirates of seven MDS patients by MACS separation. Equal numbers of CD34 positive and CD34 negative cells were lysed in Western Blot Sample Buffer and snap frozen as described previously [22]. Protein concentrations of the lysates were determined by a modified Bradford method (Bio-Rad, Munich, Germany). Cell lysates were analyzed by SDS-PAGE and Western blots with antibodies specific for CDKN1C (LSBio, Seattle, WA, USA) and  $\beta$ -actin (Cell Signaling, Leiden, The Netherlands). Western blots were quantified by the ImageJ software (version 1.6.0).

### **Immunohistochemistry**

Protein expression was determined retrospectively by immunohistochemistry in trephine biopsies of 223 patients with newly diagnosed MDS and secondary AML. Paraffin-embedded tissue sections were subjected to heat-induced epitope retrieval and used for immunohistochemical staining. A mouse monoclonal antibody against CDKN1C (clone 57P06, Thermo Scientific, Dreieich, Germany) together with an LSAB2 Kit (DakoCytomation, Hamburg, Germany) were used to detect CDKN1C protein. The nuclei were stained with hematoxylin (Merck, Darmstadt, Germany). The images were collected with a BX51 light microscope equipped with a DP50-CCD camera (both from Olympus, Hamburg, Germany). Patients were divided into two cohorts: less than one CDKN1C positive cell per high-power field (HPF, CDKN1C negative) and at least one CDKN1C positive cell per HPF (CDKN1C positive). Trephine biopsies were evaluated by two pathologists (MA, TLo) without the knowledge of the patients' clinical outcome.

## Statistical methods

The overall survival (OS) time of patients was calculated from the date of BM puncture (patients with mRNA gene expression data) or time of BM biopsy (immunohistochemistry data) to death of any cause. If more than one biopsy was available the first available biopsy was used for immunohistochemistry. Survival curve estimation was done using the method of Kaplan and Meier. Confidence interval (CI) estimation for survival curves was based on the cumulative hazard function using Greenwood's formula for variance estimation. Median follow up was estimated by the reversed Kaplan and Meier method. Patients who received allogeneic stem cell transplantation (alloSCT) were not censored at the time of alloSCT.

For MDS patients with mRNA gene expression data available (n=125), the proliferation signature score published by Starmans et al. [23] was calculated using a single representative probe set for each gene [21]. The correlation between proliferation signature scores and *CDKN1C* expression levels was estimated by Pearson's correlation coefficient. Univariable Cox regression models with respect to OS were calculated for the proliferation signature score and *CDKN1C* gene expression. For illustration purposes the Beran estimate of the survival distribution for the proliferation signature score and *CDKN1C* gene expression was used [24]. The bivariate distribution of survival times and predictor values were estimated by a nearest-neighbors method using a rectangular kernel [24,25]. Neighborhoods for each predictor value of interest were estimated according to Sheather and Jones [26] to be 18% of the closest values of each signature value in both directions. For illustration, the estimated survival curves for low, intermediate, and high levels of a predictor are depicted, determined as the survival curve estimate for the neighborhood of the smallest, median, and largest values of the predictor, respectively.

Gene sets of the KEGG pathway database (<http://www.genome.jp/kegg>) were evaluated with regard to *CDKN1C* expression and OS. This was done using the global test of Goeman et al.



[27] to test the association between sets of genes and clinical outcome. Correction of multiple testing to control the family-wise error rate was performed using the method of Holm [28]. Cell cycle and proliferation associated genes that had the strongest inverse association with OS were identified. Among these genes we choose to further validate *CDKN1C* because biological consequences of over-expression of *CDKN1C* resulting in inhibition of proliferation are well established [29]. *CDKN1C* mRNA expression was further validated and correlated with the proliferation score and the percentage of medullar blasts (less than 5% blasts, 5-9% blast and 10-19%) using the Jonckheere-Terpstra trend test. A multivariable Cox proportional hazards regression model including mRNA *CDKN1C* expression and established risk factors (BM blast count, number of cytopenias, cytogenetic risk) as covariates was applied to determine the prognostic impact of *CDKN1C* mRNA expression.

For analysis of results obtained by immunohistochemistry, numbers of Ki67 and CD34 double positive cells were compared with regard to blast counts in 10 control subjects and 24 representative MDS patients applying the Mann-Whitney test. Expression levels of CDKN1C protein in CD34<sup>+</sup> versus CD34<sup>-</sup> cells were compared using Wilcoxon's signed rank test. Patient, disease and treatment characteristics between CDKN1C positive and negative patients were compared using Fisher's exact for categorical data and the Mann-Whitney test for at least ordinal data. A Cox proportional hazards regression including CDKN1C protein expression and established risk factors (age, BM blast count, number of cytopenias, cytogenetic risk groups) was used to evaluate the prognostic impact of CDKN1C. In addition, we tested a possible interaction between CDKN1C status and subsequent treatment. Survival curves of CDKN1C positive versus CDKN1C negative patients in all patients as well as for different treatment groups were estimated by the method of Kaplan and Meier.

Statistical calculations were performed using IBM® SPSS® Statistics (Version 21.0, Chicago, USA) and the statistical software environment R, version 2.15.3, together with the R/Bioconductor packages 'jetset', version\_1.0.0, 'coin', version\_1.0-21, 'rms', version 3.5-0,

'Hmisc', version 3.9-3, globaltest', version\_5.8.1, 'pec', version\_2.1.7. All statistical tests were two-sided. Hazard ratios (HR) were estimated with 95% confidence interval (95% CI). Results with  $P$  values smaller than 5% were considered to be statistically significant.

## RESULTS

### Gene expression profiling data: pathway analysis, proliferation signature score, correlations and prognostic impact on survival

Gene expression profiling data on BM CD34<sup>+</sup> cells of MDS patients were obtained from a previous study [17]. Patient and disease characteristics are given in **Supplementary Table S1**. The estimated median follow-up time after BM aspiration was 47 months.

To test the hypothesis that cells of MDS patients are characterized by a non-proliferative phenotype, the proliferation signature score published by Starmans et al. [23] was used. Lower expression of proliferative genes strongly associated with poor prognosis (upper *versus* lower quartile of proliferation HR 0.57, 95% CI 0.43-0.77,  $P < 0.001$ ). **Figure 1A** provides the corresponding Beran estimate of the survival curve at low, intermediate and high levels of the proliferation signature score. Furthermore, the proliferation signature score was significantly lower in MDS patients as compared to controls ( $P = 0.009$ ) and showed an inverse association with BM blast counts ( $P < 0.001$ ) (**Figure 1B**).

In order to identify genes that are associated with lower proliferative activity, we performed pathway analysis using the KEGG pathway database (<http://www.genome.jp/kegg>). With the intention to later validate results of mRNA expression on the protein level, we sought to identify cell cycle relevant genes that were associated with poor survival. The search was restricted to overexpressed genes because in trephine biopsies positive staining can be captured more reliably than loss of staining. *SMAD4* and *cyclin-dependent kinase inhibitor 1C* (*CDKN1C*) were among the most significant prognostic cell cycle relevant genes that were both overexpressed and associated with poor prognosis in MDS patients (Goeman's global test;  $P < 0.001$  and  $P = 0.01$ , respectively). Since the consequences of *CDKN1C* expression resulting in inhibition of proliferation are well established [29], we choose to further

investigate and validate *CDKN1C*. It should be noted that *CDKN1C* was not part of the proliferation signature score published by Starmans et al. [23].

Higher expression levels of *CDKN1C* were associated with a lower proliferation signature score (Pearson's correlation coefficient;  $r = -0.52$ , 95% CI  $-0.63, -0.39$ ,  $P < 0.001$ ). **Figure 1C** shows the relationship between proliferation signature score and *CDKN1C* gene expression, fitted by a principal curve [30]. *CDKN1C* gene expression showed, among others, strong correlation with cell cycle pathways, DNA replication and repair, and nucleotide metabolism (**Supplementary Table S2**). Higher expression levels of *CDKN1C* were associated with lower expression levels of *MKI67* (*MKI67* is the gene name of Ki67; Pearson's correlation coefficient;  $r = -0.50$ , 95% CI  $-0.62, -0.37$ ,  $P < 0.001$ ) (**Figure 1D**). *CDKN1C* mRNA levels were significantly higher in MDS patients than in normal donors ( $P < 0.001$ ); in MDS patients *CDKN1C* expression levels were positively associated with BM blast counts ( $P = 0.004$ ) (**Figure 1E**). Accordingly, OS was shorter in patients showing high *CDKN1C* expression levels (**Figure 1F**) and multivariable Cox regression analyses confirmed *CDKN1C* expression as prognostic factor for overall survival. The hazard ratio for an increase from lower to upper quartile of the distribution of *CDKN1C* gene expression was estimated as HR 1.55 95% CI 1.09-2.23 ( $P = 0.02$ ; **Supplementary Table S3**).

### **Assessment of proliferation, *CDKN1C* mRNA and protein expression in MDS patients**

First, immunohistochemistry double staining for Ki67 and CD34 in trephine biopsies was performed in 24 representative MDS patients and 10 controls in order to provide supportive evidence that CD34<sup>+</sup> cells of MDS patients exhibit a non-proliferating phenotype (**Figure 2A**). MDS patients displaying excess BM blasts ( $\geq 5\%$ ) had a significantly lower proportion of Ki67 and CD34 double positive cells (**Figure 2B**). In order to demonstrate that *CDKN1C* expression can be reproduced in CD34<sup>+</sup> cells, fresh BM mononuclear cells of three MDS patients were sorted and *CDKN1C* mRNA expression was quantified using RT-PCR analyses.

Highest mRNA levels of *CDKN1C* were found in the CD34<sup>+</sup>CD38<sup>low</sup> cell fraction (**Figure 2C**). In Western blot analyses of 7 MDS patients CDKN1C was significantly overexpressed in the CD34<sup>+</sup> fraction (Wilcoxon's signed rank test,  $P = 0.02$ ) (**Figure 2D**). Furthermore, to confirm CDKN1C mRNA expression at the protein level CDKN1C was stained by immunohistochemistry in formalin embedded patients' BM biopsies revealing heterogeneous expression of nuclear CDKN1C protein (**Figure 2E**). When regarding CDKN1C status and levels of Ki67 and CD34 double positive cells in the BM, CDKN1C positive patients had less Ki67 and CD34 double positive cells than CDKN1C negative patients (**Figure 2F**).

#### **Prognostic impact of CDKN1C expression in the BM of newly diagnosed, treatment-naïve MDS and secondary AML patients**

To validate the prognostic relevance at the protein level, CDKN1C was stained by immunohistochemistry in an independent cohort of 223 patients with newly diagnosed MDS and secondary AML. This cohort comprises a distinct group of previously untreated patients with no overlap to the gene expression profiling cohort described above. Patient and disease characteristics and information on subsequent treatment are summarized in **Table 1**. Patient median age at the time of BM biopsy was 66 (range 20-86) years with 19% older than 75 years. Median time span between BM biopsy and start of treatment was 0.5 months (range 0-59 months). Estimated median follow up time after BM biopsy was 57 months.

A total of 139 (62%) patients were CDKN1C negative, 84 (38%) patients were CDKN1C positive. Patient and disease characteristics, particularly distribution of IPSS risk groups, as well as subsequent therapy did not differ between CDKN1C positive and CDKN1C negative patients (**Table 1**). Next, we analyzed the prognostic impact of BM CDKN1C expression on OS in the entire cohort. In univariable analysis, no significant difference in OS could be observed between CDKN1C positive and negative patients when regarding the entire patient cohort (HR 1.20, 95% CI 0.85-1.69;  $P = 0.30$ ) (**Figure 3A**). In multivariable analysis, higher

BM blasts, poor cytogenetics and advanced age were significantly associated with shorter OS in the entire patient cohort confirming their overall prognostic impact. Importantly, besides these established prognostic factors CDKN1C expression emerged as a prognostic factor for survival (HR 1.54, 95% CI 1.03-2.32;  $P = 0.04$ ) (**Table 2**).

We observed a significant interaction between CDKN1C status and treatment ( $P = 0.006$ ) (**Supplementary Table S4**). Therefore, the impact of CDKN1C status on survival was analyzed according to the subsequent treatment and not with regard to WHO diagnosis or IPSS risk. Consequently, CDKN1C expression in BM prior to treatment was associated with significantly shorter survival only among patients who received conventional chemotherapy with or without subsequent alloSCT in the course of treatment ( $P = 0.002$ ) (**Figure 3B**). In contrast, BM CDKN1C expression had no impact on OS in patients not treated with conventional chemotherapy in the course of the disease ( $P = 0.40$ ) (**Figure 3C**). In the subgroup of patients who underwent alloSCT after chemotherapy, there was a trend towards shorter OS in CDKN1C positive patients in long-term survival ( $P = 0.08$ ) (**Figure 4A**). In contrast, in patients treated with chemotherapy only, a significant impact on OS ( $P = 0.008$ ) could be observed (**Figure 4B**). Keeping in mind the sample size, in the remaining treatment subgroups (untreated, hypomethylating agents only and alloSCT without chemotherapy) CDKN1C status had no significant impact on OS ( $P = 0.21$ ,  $P = 0.37$  and  $P = 0.12$ , respectively, **Supplementary Figure S1**). For the entire subgroup of patients who underwent alloSCT median time span between BM biopsy and alloSCT was 5 months, and thus longer as compared to the median of 0.5 months from BM biopsy to start of any kind of treatment. Accordingly, when OS was calculated from time of transplant till death from any cause, CDKN1C expression in BM at diagnosis was associated with a survival disadvantage, but only in patients who received conventional chemotherapy prior to alloSCT ( $P = 0.01$ ) and not in those who did not ( $P = 0.23$ ) (**Supplementary Figure S2**).

## DISCUSSION

Long-term survival of patients suffering from higher risk MDS and secondary AML has only slightly improved over the past decades [10,31]. In particular, efficacy of conventional chemotherapy is not sustained and the majority of patients die of relapsed disease [9,32]. The same applies to alloSCT in which disease recurrence is the main cause of treatment failure [33]. Quiescence of LSC is probably one of the mechanisms underlying resistance to cell cycle-dependent cytotoxic therapy [34,35]. However, the factors which contribute to cell quiescence and possibly promote or facilitate resistance towards chemotherapy are poorly understood.

In the present study, we show that a distinct mRNA signature in the subset of CD34<sup>+</sup> BM cells, which was previously validated to characterize a non-proliferating phenotype in solid tumors [23,36], was inversely associated with BM blast counts and predicted shorter survival in patients with MDS. This observation contrasts the findings in most solid malignancies where a low proliferative signature in cancer cells was associated with better prognosis [23,36]. Based on this observation we sought to identify a candidate marker characterizing this anti-proliferative phenotype in MDS BM cells. Among the most significant, over-expressed, cell cycle relevant genes were *SMAD4* and *CDKN1C*, both of which are involved in the signalling pathway of transforming growth factor beta (TGF- $\beta$ ). *SMAD4* is one of the intracellular signalling mediators of TGF- $\beta$  and TGF- $\beta$  family members [37]. *CDKN1C* (also p57<sup>KIP2</sup> or *CDKN1C/p57<sup>KIP2</sup>*) is a member of the CIP/KIP family of cyclin dependent kinase inhibitors, which binds tightly to CyclinA- and CyclinE-CDK2 complexes and inhibits proliferation [38,39]. In solid tumors, lower *CDKN1C* expression associates with uncontrolled proliferation, dedifferentiation, cell migration and invasion [29]. Mutations of *CDKN1C* are demonstrated to cause the Beckwith-Wiedemann syndrome, a condition which also predisposes to cancer [40]. *CDKN1C* is down-regulated in the majority of solid malignancies

and although there are conflicting reports concerning its role in apoptosis, it can be regarded as a candidate tumor suppressor gene [29]. Because the function of *CDKN1C* as a negative regulator of cell proliferation is well-established, we chose to further investigate and validate *CDKN1C*.

In line with the low proliferation signature, higher *CDKN1C* mRNA expression in the subset of *CD34*<sup>+</sup> cells was associated with significantly shorter OS, and independently predicted death in patients with MDS in our study. At the protein level, a low proliferative phenotype, as assessed by *CD34*/*Ki67* double staining in trephine biopsies, also showed an inverse association with patient BM blast counts. In an independent cohort including patients with secondary AML, besides established prognostic factors, all of which are used for IPSS risk assessment, *CDKN1C* expression in the bone marrow emerged as an independent predictor of poor survival. However, the adverse prognostic impact of *CDKN1C* was treatment dependent and was only observed in patients who were treated with conventional induction-type chemotherapy, irrespective of whether or not they underwent subsequent alloSCT. This finding appears to be in agreement with the paradigm that low proliferating cells are less sensitive to chemotherapeutic drugs [14]. However, at the same time, since an oncosuppressor activity for *CDKN1C* was proposed, our findings are contrary to the data published on numerous solid tumors [29] and acute lymphocytic leukemia [41], where up-regulation of *CDKN1C* in the malignant cells is associated with improved outcome. In view of these supposedly conflicting results, several hypotheses may be entertained.

In the hematopoietic system, *CDKN1C* is the most abundant family member of the CIP/KIP family of cyclin-dependent kinase inhibitors and was shown to play a predominant role in the maintenance of adult hematopoietic stem cells (HSC), acting as a crucial brake for cycling HSC that links self-renewal to cell cycle quiescence [15,42]. Moreover, *CDKN1C* was demonstrated to be a key downstream mediator of the TGF- $\beta$  signalling pathway with *CDKN1C* up-regulation being essential for TGF- $\beta$  induced cell cycle arrest [43]. The



activation of TGF- $\beta$  was shown to be a context-dependent feature of hematopoietic regeneration, restoring quiescence after myelosuppressive chemotherapy and acting as a kind of safeguard against HSC exhaustion [44]. In addition, SMAD4 was shown to be a putative niche factor maintaining HSC quiescence during steady state with deletion of SMAD4 predisposing HSCs to exhaustion [45].

Activity of the TGF- $\beta$  signalling pathway has been implicated in the pathogenesis of MDS and has been demonstrated in the BM of patients [46,47]. Therefore, although our data fail to provide evidence for a direct and functional link between low proliferation and CDKN1C expression, our data suggest that in MDS non-proliferative stem cells accumulate during disease progression. In fact, Will et al. [48] could show that the proportion of dormant HSC within stem cell compartment of the BM increases during disease progression in MDS patients resulting in a retraction of proliferative progenitor cells. Furthermore, we hypothesize that CDKN1C mediated cell cycle quiescence, when present prior to start of treatment, constitutes one of the mechanisms allowing leukemic cells to escape anti-proliferative chemotherapy. Consequently and consistent with our results, impact on treatment outcome would be most significant in the subgroup of patients receiving chemotherapy where a non-proliferative phenotype would favour poor treatment efficacy and shorter survival.

Since resistant cells may be present at diagnosis, and disease recurrence may be driven by a subpopulation of malignant cells, stimulation of cell cycling might sensitize the hematopoietic system together with LSC to anti-proliferative chemotherapy [44,49]. On the basis of results observed in breast cancer models [50], one promising approach may be the inhibition of TGF- $\beta$  activity. However, attention must be paid as inhibition of TGF- $\beta$  signalling in MDS and AML would also affect normal hematopoiesis. The resulting supposed exhaustion of the HSC pool after myelosuppressive chemotherapy would be life-threatening except in the setting of subsequent allogeneic transplantation.

Besides its retrospective nature, the limitations of our study include patient and treatment heterogeneity and the missing functional link between *CDKN1C* expression and the proposed low-proliferating phenotype in MDS. Moreover, since CD34/*CDKN1C* double staining was not feasible in patient trephine biopsies, the possibility that *CDKN1C* is also expressed in CD34 negative cells cannot be excluded. It should also be noted that current risk assessment of MDS patients is based on the revised IPSS (IPSS-R), which was introduced in 2012 and, as compared to the initial IPSS, further refined the prognostic impact of adverse and complex cytogenetics [51]. Due to the retrospective nature, we were unable to stratify patients according to IPSS-R. However, IPSS risk groups were equally distributed in *CDKN1C* positive *versus* *CDKN1C* negative patients in our study. Importantly, both multivariable analyses revealed an adverse prognostic impact of *CDKN1C* on survival in the context of age and all risk factors used for IPSS risk assessment (i.e. blast count, cytogenetics, and number of cytopenias). Another aspect concerns the distinction between diagnostic and prognostic utility of *CDKN1C* expression. Due to the lack of accurate healthy BM controls our results do not support the use of *CDKN1C* as a diagnostic marker for MDS and secondary AML. However, since recognition of novel predictors of treatment efficiency is essential to improve patient selection, response prediction and use and sequencing of available therapies [31], *CDKN1C* may constitute a novel prognostic biomarker in newly diagnosed MDS and secondary AML patients. Assessment of *CDKN1C* expression in the BM may aid selection of patients most likely to benefit from aggressive treatment regimens, particularly since it is becoming increasingly clear that lack of treatment efficacy rather than excess toxicity is the main obstacle to cure in patients suffering from MDS and secondary AML [52]. Furthermore, the potential prognostic relevance is reflected in the fact that azacitidine treatment is unable to eradicate LSCs, whereas chemotherapy did appear to have the capacity to normalize the LSC pool in newly diagnosed high-risk MDS and AML patients [53]. However, further studies in

prospective patient cohorts are clearly required before therapy decisions can be based on CDKN1C BM expression levels.

In conclusion, we report that a low-proliferative phenotype and high CDKN1C expression in the subset of CD34<sup>+</sup> cells are associated with poor survival in MDS patients. CDKN1C expression in the BM of treatment-naïve MDS and secondary AML patients emerged as an independent prognostic factor for poor survival, particularly in patients treated with conventional anti-proliferative chemotherapy. Our findings merit further study in larger prospective patient cohorts and may supplement current prognostic/predictive tools in order to facilitate and improve risk-adapted therapy of MDS and secondary AML.

## **Acknowledgments**

We thank Michael Hess and Alexandra Hof for excellent technical assistance and Maria Gawlik for assistance in the collection of clinical data. Furthermore, we would like to acknowledge the technical assistance of Bettina Walter.

## **Authors contributions, financial support and COI statement**

AR and SD designed research, performed experiments, evaluated data, performed statistical analyses, wrote the manuscript. AB performed statistical analyses, discussed the data, wrote the manuscript. MA, TL, TG, AP, KN, TG, UG, SB, JB performed experiments, evaluated data, wrote the manuscript. PD, AH evaluated data, wrote the manuscript. TL provided idea, designed research, performed experiments, evaluated data, wrote the manuscript

Financial support: This work was supported by B.L.U.T. e.V. (Weingarten, Germany), a research grant of Celgene GmbH (Munich, Germany), by Bloodwise (United Kingdom), and by the EU's seventh framework program grant (agreement number 306240; SyStemAge).

The authors declare no conflicts of interest.

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**Table 1. Patient and disease characteristics at the time of bone marrow biopsy and information on subsequent therapy.**

Parameter	Entire patient cohort (n=223)	CDKN1C positive (n=84)	CDKN1C negative (n=139)	<i>P</i> <sup>a</sup>
Age (years), median (range)	66 (20-86)	63 (20-84)	66 (20-86)	0.38
Male gender, n (%)	147 (66)	57 (68)	90 (65)	0.66
Disease according to WHO, n (%)				1.0
RA(RS)/RCMD	66 (30)	25 (30)	41 (29)	
RAEB1/RAEB2	85 (38)	32 (38)	53 (38)	
Secondary AML	72 (32)	27 (32)	45 (32)	
Cytogenetic risk group <sup>b</sup> , n (%)				0.27
Good	79 (40)	29 (38)	50 (41)	
Intermediate	63 (32)	28 (38)	35 (28)	
Poor	54 (28)	16 (22)	38 (31)	
Missing	27	11	16	
Number of cytopenias, n (%)				0.89
0-1	84 (39)	32 (40)	52 (39)	
2-3	131 (61)	48 (60)	83 (61)	
Missing	8	4	4	
IPSS risk score, n (%)				0.99
Low	25 (16)	10 (16)	15 (15)	
Intermediate-I	46 (29)	18 (30)	28 (29)	
Intermediate-II/high	87 (55)	33 (54)	54 (56)	
NA	65	23	42	
BM blast count (%), median (range)	10 (0-90)	13 (0-90)	10 (1-90)	0.395
Subsequent treatment, n (%)				0.48
No	70 (31)	29 (35)	41 (29)	
HMA only	40 (18)	12 (14)	28 (20)	
AML chemotherapy only	38 (17)	18 (21)	20 (14)	
AlloSCT without AML chemotherapy	30 (13)	10 (12)	20 (14)	
AlloSCT after AML chemotherapy	45 (20)	15 (18)	30 (22)	
Time from BM biopsy to treatment (months), median (range)	0.6 (0-59)	0.5 (0-40)	0.6 (0-59)	0.84

Abbreviations: AlloSCT, allogeneic stem cell transplantation; AML, acute myeloid leukemia; BM, bone marrow; CDKN1C, cyclin-dependent kinase inhibitor 1C; HMA, hypomethylating agent; NA, not available; PB peripheral blood; RA, refractory anemia; RA(RS), refractory anemia with ring sideroblasts; RAEB, refractory anemia with excess blasts; RCMD, refractory cytopenia with multilineage dysplasia; WHO, World Health Organization.

<sup>a</sup>CDKN1C positive patients *versus* CDKN1C negative patients.

<sup>b</sup>According to Greenberg et al. [3] for MDS and Grimwade et al. [19] for AML.

**Table 2. Prognostic impact of CDKN1C expression in the bone marrow and established risk factors on overall survival in patients with newly diagnosed MDS and secondary AML (Cox regression analysis<sup>a</sup>).**

Variable	Effect	HR	95% CI	P
<b>Patient age</b>	5 years	1.20	1.10-1.32	< 0.001
<b>BM blast count</b>	≥10% <i>versus</i> <10%	2.90	1.92-4.40	< 0.001
<b>Cytogenetic risk<sup>b</sup></b>	poor <i>versus</i> intermediate/good	1.77	1.18-2.67	0.006
<b>Number of cytopenias</b>	≥2 <i>versus</i> <2	1.42	0.95-2.12	0.09
<b>CDKN1C status</b>	positive <i>versus</i> negative	1.54	1.03-2.32	0.04

Abbreviations: AML, acute myeloid leukemia; BM indicates bone marrow; CDKN1C, cyclin-dependent kinase inhibitor 1C; CI, confidence interval; HR hazard ratio; MDS, myelodysplastic syndrome.

<sup>a</sup>Complete case analysis (n=188). <sup>b</sup>According to published criteria for MDS [3] and AML [19].

## FIGURE LEGENDS

### **Figure 1. Low-proliferation phenotype and higher *CDKN1C* expression in the subset of CD34<sup>+</sup> bone marrow cells were associated with poor survival in MDS patients.**

(A) On the basis of mRNA expression profiling data on 125 MDS patients the proliferation signature score published by Starmans et al. [2008] were calculated and correlated with overall survival using a Cox proportional hazards regression model. MDS patients displaying a lower proliferation signature scores had a worse overall survival (upper *versus* lower quartile of proliferation HR 0.57, 95% CI 0.43-0.77,  $P < 0.001$ ). For illustration purpose the Beran estimate of the survival distribution for high (dashed line), intermediate (dash-dotted line) and low levels (solid line) of the proliferation signature score was applied.

(B) The proliferation signature score was significantly lower in MDS patients ( $n = 125$ ) as compared to healthy controls ( $n = 17$ ,  $P = 0.009$ ) and showed an inverse association with bone marrow (BM) blast counts ( $P < 0.001$ ).

(C) Higher *CDKN1C* expression levels were associated with a lower proliferation signature score (Pearson's correlation coefficient;  $r = -0.52$ , 95% CI -0.63, -0.39,  $P < 0.001$ ). The solid line represents the principal curve estimate for the relationship between proliferation signature score and *CDKN1C* gene expression.

(D) Higher expression levels of *CDKN1C* were associated with lower expression levels of *MKI67* (*MKI67* is the gene name of Ki67; Pearson's correlation coefficient;  $r = -0.50$ , 95% CI -0.62, -0.37,  $P < 0.001$ ).

(E) *CDKN1C* mRNA expression levels in the subset of CD34<sup>+</sup> cells were higher in MDS patients ( $n = 125$ ) as compared to healthy controls ( $n = 17$ ,  $P < 0.001$ ) and showed a positive association with BM blast counts in MDS patients ( $P = 0.004$ ).

(F) When MDS patients ( $n = 125$ ) were stratified according to *CDKN1C* expression levels, survival was shorter in patients showing high *CDKN1C* expression levels (HR for an increase

from lower to upper quartile of the distribution 1.55 95% CI 1.09-2.23,  $P = 0.02$ , cf. **Supplementary Table S3**). For illustration purpose the Beran estimate of the survival distribution for high (solid line), intermediate (dash-dotted line) and low *CDKN1C* expression levels (dashed line) was applied.

**Figure 2. Proliferation assessment and *CDKN1C* mRNA and protein expression in MDS patients.**

**(A)** Representative immunofluorescence image showing Ki67 and CD34 double staining in a trephine biopsy. The following numbers indicate distinct cell types: 1.  $CD34^+Ki67^+$ , 2.  $CD34^+Ki67^-$ , 3.  $CD34^-Ki67^+$ .

**(B)** Results of immunofluorescence Ki67 and CD34 double staining in trephine biopsies of 24 representative MDS patients (BM blast count <5%:  $n = 6$ ; BM blast count 5-10%:  $n = 5$ ; and BM blast count 11-20%:  $n = 13$ ) and 10 controls. Numbers of  $Ki67^+CD34^+$  cells were correlated with bone marrow (BM) blast counts of the same patient. MDS patients displaying excess of BM blasts ( $\geq 5\%$ ) had significantly lower Ki67 and CD34 double positive cells ( $*P < 0.01$ ).

**(C)** Fresh BM mononuclear cells of three representative MDS patients were sorted according to their CD34 and CD38 expression levels and *CDKN1C* mRNA expression was quantified using RT-PCR analyses. Highest levels of *CDKN1C* were expressed in the  $CD34^+CD38^{low}$  cell fraction. Values were given as number of *CDKN1C* transcripts per 1000 housekeeping gene transcripts.

**(D)** Western blot analyses were performed on sorted  $CD34^+$  and  $CD34^-$  BM cells of representative MDS patients (3 patients depicted). *CDKN1C* expression was significantly stronger in  $CD34^+$  cells as compared to  $CD34^-$  cells of the same patients ( $n = 7$ ,  $P = 0.02$ ).

**(E)** Representative immunohistochemistry image showing CDKN1C protein expression in a trephine biopsy. Black arrows indicate CDKN1C positive cells.

(F) Numbers of Ki67 and CD34 double positive cells were correlated with CDKN1C status in trephine biopsies of the 24 representative MDS patients (BM blast count <5%: n = 6; BM blast count 5-10%: n = 5; and BM blast count 11-20%: n = 13). CDKN1C positive patients (n = 12) had lower levels of Ki67 and CD34 double positive cells than CDKN1C negative patients (n = 12) (\* $P = 0.04$ ).

**Figure 3. Prognostic impact of bone marrow CDKN1C protein expression on the outcome of newly diagnosed MDS and secondary AML patients.**

(A) In the entire patient cohort (n = 223) no significant difference in overall survival (OS) could be observed between CDKN1C positive (n = 84) and CDKN1C negative (n = 139) patients (univariate,  $P = 0.30$ ).

(B) In the subgroup of patients treated with conventional chemotherapy with or without subsequent alloSCT (n = 83), CDKN1C expression in the bone marrow (BM) prior to treatment was associated with significantly shorter survival with a 2-year OS of 30% (95% CI 14-45) *versus* 58% (95% CI 44-71) in CDKN1C positive (n = 33) *versus* CDKN1C negative (n = 50) patients, respectively ( $P = 0.002$ ). The median bone marrow blast count and the frequencies of AML cases did not differ in CDKN1C positive *versus* CDKN1C negative patients ( $P = 0.27$  and  $P = 0.25$ , respectively).

(C) In patients not treated with conventional chemotherapy in the course of the disease (n = 140) BM CDKN1C status (positive, n = 51; negative, n = 89) had no impact on OS ( $P = 0.40$ ). The median bone marrow blast count and the frequencies of AML cases did not differ in CDKN1C positive *versus* CDKN1C negative patients ( $P = 0.98$  and  $P = 0.35$ , respectively).

**Figure 4. Prognostic impact of bone marrow CDKN1C protein expression on the outcome of newly diagnosed MDS and secondary AML patients receiving conventional chemotherapy with and without subsequent allogeneic stem cell transplantation.**

**(A)** In the subgroup of patients who were treated with chemotherapy and subsequent alloSCT (n = 45) a trend towards shorter survival could be observed with a 2-year OS of 39% (95% CI 13-66) *versus* 63% (95% CI 46-80) in CDKN1C positive (n = 15) *versus* CDKN1C negative (n = 30) patients, respectively ( $P = 0.08$ ).

**(B)** In the subgroup of patients treated with chemotherapy only (n = 38), CDKN1C expression in the bone marrow prior to treatment was associated with significantly shorter survival with a 2-year OS of 22% (95% CI 3-41) *versus* 50% (95% CI 28-72) in CDKN1C positive (n = 18) *versus* CDKN1C negative (n = 20) patients, respectively ( $P = 0.008$ ).

## **Supplementary information**

**Supplementary Table S1.** Patient characteristics of MDS patients for whom analysis of gene expression profiling data of bone marrow CD34<sup>+</sup> cells was performed.

**Supplementary Table S2.** Global test of the correlation of mRNA expression of *CDKN1C* with relevant cell cycle pathways.

**Supplementary Table S3.** Prognostic impact of *CDKN1C* mRNA expression and established covariates on overall survival (Cox regression analysis).

**Supplementary Table S4.** *CDKN1C* bone marrow status, subsequent treatment and impact on overall survival.

**Supplementary Figure S1.** Prognostic impact of bone marrow *CDKN1C* protein expression on the outcome of newly diagnosed MDS and secondary AML patients receiving: no treatment, hypomethylating agents only, and allogeneic stem cell transplantation without prior chemotherapy.

**Supplementary Figure S2.** Prognostic impact of bone marrow *CDKN1C* protein expression at diagnosis on survival after allogeneic stem cell transplantation.