IDH2 mutations in patients with normal karyotype AML predict favorable responses to daunorubicin, cytarabine and cladribine regimen

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Mutations in isocitrate dehydrogenase 1 and 2 (IDH1/2) genes occur in about 20% patients with acute myeloid leukemia (AML), leading to DNA hypermethylation and epigenetic deregulation. We assessed the prognostic significance of IDH1/2 mutations (IDH1/2+) in 398 AML patients with normal karyotype (NK-AML), treated with daunorubicine + cytarabine (DA), DA + cladribine (DAC), or DA + fludarabine. IDH2 mutation was an independent favorable prognostic factor for 4-year overall survival (OS) in total NK-AML population (p = 0.03, censoring at allotransplant). We next evaluated the effect of addition of cladribine to induction regimen on the patients’ outcome according to IDH1/2 mutation status. In DAC group, 4-year OS was increased in IDH2+ patients, compared to IDH- wild type group (54% vs 33%; p = 0.0087, censoring at allotransplant). We next evaluated the effect of addition of cladribine to induction regimen on the patients’ outcome according to IDH1/2 mutation status. In DAC group, 4-year OS was increased in IDH2+ patients, compared to IDH-wild type group (54% vs 33%; p = 0.0087, censoring at allotransplant), while no difference was observed for DA-treated subjects. In multivariate analysis, DAC independently improved the survival of IDH2+ patients (HR = 0.6 [0.37–0.93]; p = 0.024; censored at transplant), indicating that this group specifically benefits...
Mutations in isocitrate dehydrogenase 1 and 2 (IDH1/2) genes are observed in up to 20% patients with acute myeloid leukemia (AML) and constitute an early clonal event in the evolution of this disease. The most common IDH2 mutations in AML involve arginine 140 and 172 (R140 and R172) residues, which account for over 80% of all mutated IDH2 cases. IDH1 mutations occur less frequently than IDH2 in total AML population (7.7% for IDH1 vs 15.4% for IDH2) and lead to a substitution of histidine or cysteine (R132H and R132C). All mentioned pathogenic IDH1/2 mutations occur at the conserved active site of the enzymes and endow mutant enzymes with a neomorphic activity, converting alpha-ketoglutarate (αKG) to 2-hydroxylglutarate (2HG). Accumulation of 2HG competitively inhibits the activity of αKG-dependent enzymes, including Tet methylcytosine dioxygenase 2 (TET2), engaged in DNA hydroxymethylation and histone demethylation. Thus, AML cells with IDH1/2 mutations are characterized by unique hypermethylated DNA signature, which results in blocked hematopoietic differentiation.

The prognostic implications of somatic IDH mutations in patients with normal karyotype AML (NK-AML) remain controversial. Although the co-existent aberrations, such as nucleophosmin 1 (NPM1) mutation and internal tandem duplication of fms-like tyrosine kinase 3 (FLT3-ITD), have a clear impact on clinical aggressiveness of IDH1/2-mutated (IDH1/2+) leukemias, even in a selected NPM1/FLT3-ITD NK-AML subpopulation, the prognostic impact of IDH1/2 mutations is still very heterogenous, and the factors responsible for such prognostic discrepancies are not fully understood. Since there are apparent differences in the treatment protocols between independent trials, different induction regimens might explain these conflicting results.

Addition of a purine analogue cladribine to daunorubicin + cytarabine 3 + 7 protocol (DA + cladribine; DAC) is an established modification of standard AML induction regimen, supported by published clinical trials from the Polish Adult Leukemia Group (PALG) and the international STAMP2 studies. The activity of cladribine has been mostly attributed to increased bioactivation of AraC in leukemic blasts as well as direct inhibition of DNA synthesis. Importantly, cladribine exhibits DNA hypomethylating activity due to its ability to inhibit S-adenosylhomocysteine hydrolase (SAHH) and to reduce the pool of active methyl donor S-adenosylmethionine (SAM) in leukemic cells. Our group has demonstrated in previous PALG studies that DAC was associated with increased complete remission (CR) rate and prolonged overall survival (OS), with the most significant benefit in patients with unfavorable cytogenetics. Recently, we have also shown that the addition of cladribine alleviated the negative effect of FLT3-ITD on the CR rate and OS in NK-AML patients.

Given the profound metabolic and epigenetic consequences of IDH1/2 mutations and cladribine hypomethylating properties, we hypothesized that IDH1/2 mutant leukemia blasts may exhibit differential sensitivity to DA and DAC induction regimens. In the current study, we demonstrate that DAC induction is associated with statistically significant improvement of outcome in IDH2+ NK-AML patients when compared to standard DA regimen. Finally, we postulate that this beneficial effect toward IDH2+ NK-AML results from the hypomethylating activity of cladribine. With ongoing clinical studies on IDH1/2 inhibition combined with high-intensity induction regimen for newly diagnosed AML, our data suggest that cladribine might be a potent combination partner for multi-agent therapy of IDH2+ AML patients.

Results

Prognostic relevance of IDH1/2 mutations in the entire NK-AML population and subgroups according to NPM1/FLT3 mutational status.

Of the 398 analyzed de novo NK-AML cases, 80 (20.1%) patients had missense IDH1/2 mutations (IDH1/2*). Among the IDH1/2* subgroup, 30 (37.5%) subjects carried IDH1 mutations in the R132 position. Of the 50 IDH2+ patients, 35 (43.75%) and 15 (18.75%) patients carried mutations in the R140 and R172 position, respectively. The median follow-up was 40.8 months and the median survival reached 18.8 months. The estimated 4-year survival for the whole group was 37.5% with statistically significant improvement of outcome in NPM1/FLT3-ITD+ NK-AML patients when compared to standard DA regimen. Finally, we show that this beneficial effect toward IDH2+ NK-AML results from the hypomethylating activity of cladribine. With ongoing clinical studies on IDH1/2 inhibition combined with high-intensity induction regimen for newly diagnosed AML, our data suggest that cladribine might be a potent combination partner for multi-agent therapy of IDH2+ AML patients.
Table 1. Patients characteristics. alloHSCT, allogeneic hematopoietic stem cell transplantation; CR1, first complete remission; F, female; M, male; FAB, French American British classification; WBC, white blood cells; * calculated using the U-Mann Whitney test; ** computed by the Fisher exact test or Chi square; †† for 29 patients information on FAB status was not established; ‡‡ for 2 high and 1 low risk NK-AML patients missing IDH2 mutation analysis (one received DAC and the remaining patients—DA); for 3 patients D4F status was not established; ap = 0.08 when comparison was done for R172 IDH2 mutation status was not established; †† for 29 patients information on FAB status is lacking: *p = 0.08 when comparison was done for R172 IDH2 versus IDH1/2 patients restricted to NPM1− / FLT3-ITD− subgroup.

| Total no | Total no | Total no | Total no | IDH1 R132* | IDH2 R140* | IDH2 R172* | IDH1/2* | IDH1/2* | IDH1/2* | P value: IDH1* versus IDH1/2* | P value: IDH2* versus IDH1/2* | P value: R172 IDH2* versus IDH1/2* |
|----------|----------|----------|----------|------------|------------|------------|----------|----------|----------|------------------------------|------------------------------|-------------------------------|
| NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) | NPM1+/FLT3-ITD− (n = 398†) |
| Median age (years) | 50 | 56 | 50 | 55 | 49 | 0.02 | 0.13 | 0.2* |
| < 50 years | 207 (52%) | 9 (30%) | 18 (51.4%) | 3 (20%) | 174 (55%) | 0.007 | 0.7 | 0.0073 |
| ≥ 50 years | 191 (48%) | 21 (70%) | 17 (48.6%) | 12 (80%) | 141 (45%) | - | - | - |
| NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) | NPM1+/FLT3-ITD−/C-CEBPA (n = 398†) |
| Median initial WBC (x10^9/L) | 64.1 | 77.7 | 23.7 | 2.1 | 73.0 | 0.56 | 0.0034 | 0.000003 |
| Sex** | F | 221 (55.5%) | 26 (52%) | 20 (50%) | 20 (50%) | 244 (56%) | 0.6 | 0.47 | 0.84 |
| M | 177 (44.5%) | 24 (48%) | 8 (53%) | 7 (47%) | 193 (44%) | - | - | - |
| FAB***†† | M0 | 20 (5.2%) | 11 (37%) | 12 (40%) | 15 (43%) | 15 (100%) | 0.47 | 0.66 | 0.0001 |
| M1 | 80 (20.8%) | 9 (30%) | 7 (20%) | 0 (0%) | 19 (6.2%) | 0.07 | 0.01 | 0.044 |
| M2 | 132 (34.4%) | 11 (37%) | 13 (37%) | 7 (46.65%) | 19 (18.8%) | 0.3 | 0.1 | 0.15 |
| M4 | 98 (25.5%) | 7 (23%) | 10 (28.6%) | 1 (6.7%) | 22 (7%) | 0.5 | 0.5 | 0.03 |
| M5 | 51 (13.3%) | 3 (10%) | 3 (8.6%) | 1 (3%) | 21 (6.7%) | 0.056 | 0.23 | 0.093 |
| M6 | 5 (1.3%) | 0 (0%) | 1 (3%) | 0 (0%) | 7 (46.65%) | 0.056 | 0.23 | 0.093 |
| Induction** | DA | 191 (48%) | 11 (36.6%) | 20 (57%) | 8 (53%) | 150 (47.6%) | 0.25 | 0.28 | 0.66 |
| DAC | 176 (44%) | 17 (56.7%) | 11 (31.4%) | 7 (47%) | 140 (44.4%) | 0.2 | 0.14 | 0.86 |
| DAF | 31 (8%) | 6 (23%) | 4 (11.6%) | 0 (0%) | 25 (8%) | 0.016 | 0.5 | 0.016 |
| 2nd induction | 135 (36%) | 6 (23%) | 14 (40%) | 7 (47%) | 108 (36.6%) | 0.02 | 0.32 | 0.26 |
| Time to alloHSCT* (days) | 321 | 493 | 250 | 605 | 305 | 0.06 | 0.88 | 0.001 |
| AlloHSCT in CR1** | 126 (32%) | 3 (10%) | 10 (28.6%) | 5 (33%) | 108 (34.3%) | 0.0035 | 0.5 | 0.59 |

**Table 2. Impact of the addition of cladribine to standard DA induction on the outcome of IDH1/2* NK-AML patients.** Further we compared the clinical outcome of DAC vs DA treated patients. The DAC induction was associated with improved 4-year OS in high risk IDH2* patients compared to standard DA regimen after censoring for HSCT (OS: 50% vs 13% respectively; p = 0.04; Fig. 3A,B, Supplemental Table S2). Specifically, the addition of cladribine resulted in improved OS for IDH2* patients in the NPM1+/FLT3-ITD− subgroup (HR:0.3; 95% CI 0.08–0.95; p = 0.04), but not for IDH2* or IDH1* patients (Fig. 3C,D, Supplemental...
Table S2). The favorable effect of cladribine on outcome in IDH2+ subgroup was limited to younger patients (< 50 years) (Supplemental Fig. S3). However, in multivariate analysis for IDH2+ patients, DAC induction was independently associated with reduced risk of death when the observations were censored at alloHSCT (HR: 0.21; 95% CI 0.056–0.8; p = 0.02; Table 3).

Hypomethylating activity of cladribine as a possible mechanism leading to improved survival of IDH2+ NK-AML patients. Since our analyses indicated that cladribine was associated with improved outcomes for IDH2+ patients, we further investigated possible biological mechanisms underlying this phenomenon. Mutations in IDH2 endow the enzyme with the neomorphic activity to produce 2-hydroxyglutarate (2HG), which functions as a competitive inhibitor of 2-ketoglutarate-dependent enzymes, such as TET2, a DNA-demethylating enzyme 5. We therefore investigated, whether cladribine could limit 2HG-dependent DNA hypermethylation in AML cells. To this end, HEL and MOLM14 cell lines were treated with synthetic cell-permeable derivative of 2HG, octyl-2HG, alone or in combination with cladribine for 24 h. For these experiments, we used low cladribine doses (10 nM and 25 nM), which were non-toxic to the cells over the 24 h treatment period (Supplemental Fig. S4). Octyl-2HG significantly increased DNA methylation, measured by 5-methylcytosine abundance, whereas simultaneous addition of cladribine suppressed DNA hypermethylation (Fig. 4A). We next tested the hypomethylating effect of cladribine in HEL cells overexpressing IDH2 R140 and IDH2 R172 mutants.

Table 2. Multivariate analysis for different genetic subgroups of total NK-AML patients. All treatment groups (DA, DAC, DAF) were included in the analysis. CR, overall complete remission rate after all courses of inductions; OS, overall survival; allo OS, overall survival censored at allograft; RFS, cumulative incidence of relapse; SD, standard deviation; HR, hazard ratio; OR, odds ratio; CI, confidence interval. † for whole NK-AML cohort: 3 patients missing IDH2 mutation analysis (2 of IDH2 missing patients were HR NK-AML. 1 was LR); 3 patients missing classification according to NPM1/FLT3-ITD status; # computed by log rang test; ** computed by Chi square or Fisher exact test; * computed by logistic regression analysis; **Computed by Cox regression analysis.
Figure 1. Kaplan–Meier estimates for the probability of overall survival of (A) total NK-AML population, as well as (B) high-risk and (C) low-risk subgroups according to IDH1 and IDH2 mutational status. In (A) and (B) data were censored at the time of alloHSCT. OS—overall survival, HR—high-risk AML, LR—low-risk AML; n—number of patients, p—p value.
Figure 2. Impact of IDH2 mutation status on survival in DAC and DA treated subgroups. (A) IDH2+ mutations have a positive impact on the survival of patients treated with DAC regimen. (B) Lack of difference in OS between IDH2+ and IDH2− patients in DA group. OS with observations was censored at time of allo HSCT; n—number of patients, p—p value.
The overproduction of 2HG in generated IDH2 mutant cell lines was confirmed by liquid chromatography-mass spectrometry analyses (Supplemental Fig. S5). As expected, cells with IDH2 R140 and IDH2 R172 mutations induced DNA hypermethylation, comparing to IDH2 wild type (IDH2wt) cells (Fig. 4B). Incubation of cells overexpressing IDH2-mutants with cladribine (10 nM or 25 nM, 24 h) decreased 5-methylcytosine levels comparably to the IDH2-R140-specific inhibitor AGI-6780 (Fig. 4B). Of note, combination of cladribine with AGI-6780 further decreased DNA methylation, as compared to the either compound used alone (Supplemental Fig. S6). Although introduction of IDH1 R132H mutation induced 2-HG production, the global DNA methylation level did not differ between the mutant and wild type cells, and remained unchanged after addition of cladribine or IDH1 R132H-targeting inhibitor (AGI-5198), (Supplemental Fig. S7). At low doses, cladribine inhibits the activity of S-adenosylhomocysteine hydrolase, a key enzyme in the biosynthesis pathway of S-adenosylmethionine (SAM), which constitutes a methyl group donor in DNA methylation reactions18–20,35. Therefore we determined, whether cladribine compromises DNA methylation by affecting the cellular SAM level. Consistent with our hypothesis, incubation of HEL cells overexpressing IDH2 mutants with cladribine decreased SAM pool without influencing 2HG production, in contrast to AGI-6780, which reduced 2HG without affecting the SAM level (Fig. 4C,D).

**Discussion**

The prognostic significance of IDH1/2-mutations in patients with NK-AML is controversial, with conflicting reports in the literature2,8,9,12,36,37. In the present study, we report that the impact of IDH2 mutations on patient outcomes was related to the specific regimen used: the addition of cladribine to standard daunorubicin and cytarabine (DA) induction was independently associated with longer survival for IDH2+ patients (after censoring...
observations at alloHSCT). Our findings suggest that the mechanism for this beneficial effect is related to cladr

In our study, the IDH2-R140 mutation was associated with superior outcomes in the entire NK-AML, uniquely when accompanied by NPM1 mutations, confirming the previous results. Interestingly, this effect was not only NPM1 mutation-dependent, but also IDH2-specific: we found the favorable effect of NPM1 mutations only in patients with co-occurring IDH2 mutations, suggesting synergy between the two mutations. Neither IDH2-R140 nor R172 impacted outcomes of patients in NPM1−/FLT3-ITD− subgroup. These data are similar to the findings of Patel et al., but different from other studies reporting a poor or uniquely favorable impact of the IDH2 R172 mutation on prognosis. These discrepancies may be related to study inclusion criteria, type of IDH1/2 mutation, age, disease history as well as cytogenetic background of the analyzed population. In addition, recent high-throughput sequencing studies have shown that de novo IDH1/2− NK-AML frequently coexist with adverse risk-associated mutations in DNMT3A, ASXL1, RUNX1, SRSF2, PHF6. These discrepancies may be related to study inclusion criteria, type of IDH1/2 mutation, age, disease history as well as cytogenetic background of the analyzed population.

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The effect of specific treatment has not been evaluated in the previous reports concerning the prognostic significance of IDH1/2 mutations. In our study, two high-intensity induction regimens: daunorubicin + cytarabine (DA) versus daunorubicin + cytarabine + cladribine (DAC) were used to treat NK-AML patients. Our analysis showed that the addition of cladribine was associated with significantly improved outcomes in IDH2−mutated patients. In the NPM1+/FLT3-ITD− genotype, both IDH2 R140 and R172 mutations showed favorable

Table 3. Results of multivariate analysis restricted for IDH2+ patients in different genetic subgroups of NK-AML patients. CI, confidence interval; CR, complete remission; HR, hazard ratio; OR, odds ratio; OS, overall survival; χ2 computed by Chi square or Fisher exact test; log rank test, * computed by logistic regression analysis, ** computed by Cox regression analysis.

| End point and variables                        | Total NK-AML (n = 50) | Molecular higher risk: NPM1−/FLT3-ITD+ and FLT3-ITD+ (n = 37) |
|-----------------------------------------------|-----------------------|---------------------------------------------------------------|
|                                              | CR rate after 1st induction OR (95% CI) | CR rate after 1st induction OR (95% CI)                      |
|                                              | Age (continuous)       | Age (continuous)                                              |
|                                              | 0.97 (0.92–1.033)      | 0.99 (0.92–1.05)                                             |
|                                              | CEBPA double mut        | 1.022 (0.31–3.35)                                            |
|                                              | 1.06 (0.33–3.4)        | 1.26 (0.1–14.96)                                             |
|                                              | NPM1 mut               | 0.25 (0.067–0.92)                                            |
|                                              | 1.77 (0.12–26.18)      | 3.2 (0.578–17.54)                                            |
|                                              | DMC versus DA          | 2.04 (0.49–8.41)                                             |
|                                              | 4-year OS HR (95% CI)  | 0.39 (0.14–1.1)                                              |
|                                              | Age (continuous)       | 1.04 (0.99–1.09)                                             |
|                                              | CEBPA double mut        | 1.8 (0.2–15.3)                                               |
|                                              | NPM1 mut               | 0.25 (0.067–0.92)                                            |
|                                              | FLT3-ITD               | 0.25 (0.067–0.92)                                            |
|                                              | DMC versus DA          | 1.26 (0.1–14.96)                                             |
|                                              | 4-year OS censored at allograft HR (95% CI) | 0.39 (0.14–1.1) |
|                                              | Age (continuous)       | 1.03 (0.97–1.1)                                              |
|                                              | CEBPA double mut        | 1.6 (0.18–13.7)                                              |
|                                              | NPM1 mut               | 0.18 (0.035–0.87)                                            |
|                                              | FLT3-ITD               | 0.18 (0.035–0.87)                                            |
|                                              | DMC versus DA          | 2.12 (0.41–11.06)                                            |
|                                              | 4-year OS censored at allograft HR (95% CI) | 0.21 (0.056–0.8) |
|                                              | Age (continuous)       | 1.04 (0.98–1.1)                                              |
|                                              | CEBPA double mut        | 1.8 (0.2–15.3)                                               |
|                                              | NPM1 mut               | 0.79 (0.22–2.87)                                             |
|                                              | FLT3-ITD               | 0.79 (0.22–2.87)                                             |
|                                              | DMC versus DA          | 0.4 (0.14–1.15)                                              |
|                                              | 4-year OS censored at allograft HR (95% CI) | 0.21 (0.056–0.8) |
|                                              | Age (continuous)       | 1.04 (0.98–1.1)                                              |
|                                              | CEBPA double mut        | 1.39 (0.16–12.03)                                            |
|                                              | NPM1 mut               | 0.18 (0.016–2.07)                                            |
|                                              | FLT3-ITD               | 0.18 (0.016–2.07)                                            |
|                                              | DMC versus DA          | 0.15 (0.03–0.77)                                             |
|                                              | 4-year OS censored at allograft HR (95% CI) | 0.02 ** |

observations at alloHSCT). Our findings suggest that the mechanism for this beneficial effect is related to cladr

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Figure 4. Cladribine decreases IDH2 mutation-induced DNA hypermethylation. (A) Cladribine decreases DNA hypermethylation induced by incubation of HEL and MOLM14 AML cell lines with synthetic derivative of 2HG (octyl-2HG). (B) Cladribine restrains DNA hypermethylation induced by overexpression of IDH2 R140Q and R172K mutants. (C) Cladribine reduces SAM level in IDH2-mutant AML cells. (D) In contrast to IDH2-mutant inhibitor AGI-6780, cladribine does not change the level of 2HG in cells overexpressing IDH2 R140Q and IDH2 R172K. For A and B representative histograms from 3 independent experiments were shown. Graphs in C and D show mean ± standard deviation from 3 independent experiments. *** for p < .001; ** for p < .01 and * for p < .05. Statistics was calculated with unpaired T-test.
impact in the DAC-treated group, suggesting that the effect was IDH2-specific. Neither IDH2 R140 nor IDH2 R172 mutations were prognostic in the DA-treated subgroup, consistent with Patel et al. Multivariate analysis identified cladribine as an independent prognostic factor for longer survival for IDH2+ patients in both the entire NK-AML cohort and the NPM1+/FLT3-ITD+ subgroup. Thus, cladribine may be beneficial both in IDH2+ and FLT3-ITD+ leukemias.

Intriguingly, the favorable effect of cladribine in the IDH2-mutated cohort was significant only when censoring for alloHSCT in most of the analyses. Therefore it is possible, that the impact of cladribine in IDH2+ patients is overshadowed in the setting of alloHSCT, e.g. due to improved survival of transplanted IDH2+ patients. Our data may also suggest that early alloHSCT in IDH2+ patients does not offer an advantage over chemotherapy, as has been observed for NPM1+ patients. These possible explanations are further being investigated in an ongoing, prospective randomized clinical trial.

The mechanism of sensitivity of IDH2-mutant cells to cladribine is unknown. Our data show that in cells overexpressing IDH2-mutants, cladribine decreased SAM levels and DNA cytosine methylation, with no impact on 2HG production. Thus, in IDH2-mutant cells, cladribine may deplete the methyl donor pool, impair methylation reactions, and lead to decreased global DNA methylation, despite sustained production of 2HG and ongoing inhibition of 2HG-dependent enzymes, including DNA demethylases. Importantly, as concentrations similar to those used in our in vitro studies are achieved clinically using the standard doses of cladribine, corresponding levels of demethylating activity likely also occur in vivo. Thus, cladribine and IDH2 inhibitors may have different, and potentially synergistic mechanisms of DNA demethylation and our preliminary in vitro data confirmed the synergy between cladribine and IDH2 R140Q-specific AGI-6780. Importantly, in the light of our findings, cladribine could be an interesting treatment alternative in patients with \(\text{trans or cis} \) resistance to IDH2 inhibition.

Although both IDH2+ and IDH1+ mutations are reported to overproduce 2HG, in our study cladribine did not improve the survival of patients with IDH1 mutations. Despite parallel mechanisms of transformation, IDH1+ and IDH2+ leukemias show differences in both in vitro and clinical studies. This discrepancy might be related to distinct cellular localization of IDH1 and IDH2 molecules (cytoplasmic vs mitochondrial), followed by various downstream metabolic consequences, including differential response to cytotoxic drugs. In our in vitro IDH overexpressing model, global DNA hypermethylation was attributed only to IDH2 mutations, but not to IDH1 R132H cells. To support, although DNA hypermethylation was previously reported in both IDH1 and IDH2-mutants overexpressing HEK293T cells, 5-methylcytosine level was considerably lower in IDH1 than IDH2-mutants. Furthermore, IDH1+ and IDH2+ leukemias differ in their mutational profiles, with high incidence of DNA (cytosine-5)-methyltransferase 3A (DNMT3A) mutations reported in IDH1+, but not IDH2-R140+ AML. As DNMT3A mutations impact DNA methylation profile, it is very likely, that their co-segregation with IDH1 mutations might change the response to cladribine.

In summary, our data show that the addition of cladribine to standard AML induction therapy resulted in improved outcomes in patients with IDH2 mutation. The mechanism of this synthetic effect likely involves cladribine's demethylating activity in a molecular background of the mutation-induced DNA hypermethylation. Given the limitations of this study (retrospective nature, lack of comprehensive mutational profile at diagnosis, and relatively small IDH1/2+ subgroups), further investigations on cladribine as a treatment option for IDH1/2+ patients are warranted. Of note, a randomized, international study comparing DA versus DAC regimens has been already launched, with complete remission, overall survival and multimodality assessments of measurable residual disease as the study endpoints.

**Patients and methods**

**Patients characteristics, material collection and molecular tests.** A total of 398 de novo NK-AML patients treated in 9 PALG centers between 1999 and 2014 were either prospectively randomized to 1 of the 3 treatment groups (in the years 2000–2006): daunorubicin + cytarabine (DA; \(n = 18\)), daunorubicine + cytarabine + cladribine (DAC; \(n = 24\)), daunorubicine + cytarabine + fludarabine (DAF; \(n = 20\)), or treated outside the trial (2006–2014), according to DA (\(n = 173\)), DAC (\(n = 152\)) or DAF (\(n = 11\)) induction protocols, at the discretion of the treating physician (Table 1, Supplemental Table S3). Of note, fewer patients were included from years 2000–2006 due to limited access to molecular genetic data. Analysis of the prognostic significance of IDH1/2 mutations was performed for the entire population (DA-, DAC- and DAF-treated; Supplemental Table S2), while the impact of cladribine on outcomes of the IDH2+ NK-AML population was evaluated in the DAC- vs DA-treated groups (Table 3 and Supplemental Table S3). All patients included in the study were eligible for intensive induction treatment with the age range from 18 to 76 years and median age of 50 years. All samples were obtained with written informed consent, in accordance with the Declaration of Helsinki. The study was approved by the local Bioethics Committees of Warsaw Medical University for all participating institutions. The mutation status of IDH1/2 was determined as previously described. Details of the material collection and molecular tests are described in Supplementary Figures and Information.

**Treatment protocols.** DA consisted of daunorubicine 60 mg/m² as a 5-min infusion on days 1 through 3 and a continuous infusion of cytarabine 200 mg/m² on days 1 through 7. DAC additionally included cladribine (5 mg/m²) administered as a 3-h infusion on days 1–5, while the DAF regimen consisted additionally of fludarabine 25 mg/m² administered on days 1–5. Second courses of induction were permitted at the discretion of the treating investigator. Post-remission therapy protocols were comparable in all induction groups, including rates of alloHSCT (DAC, 32%; DA, 36.6%; \(p = 0.37\)). The data on IDH1/2 mutation status and induction protocol for patients who went to transplant are given in Table 1 and Supplemental Table S1.
Overall survival was defined as time from diagnosis to either death or last observation alive. Data analyses and Information.

The statistical analyses were performed using STATISTICA 12 (StatSoft Inc. Tulsa, OK, USA).

we used CR-based analysis. For comparison of CR rates or frequency distribution of other characteristics between subgroups, we used Chi-square or Fisher exact test (when the number of patients per subgroup was < 5). In multivariate analyses logistic regression and Cox proportional model were used to compare CR rates and OS, respectively. The statistical analyses were performed using STATISTICA 12 (StatSoft Inc. Tulsa, OK, USA).
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Author contributions

M.L., P.J., S.G., A.W., I.H., O.H. project design; M.P., P.G., K.B., M.W., I.F., K.M., B.J., I.S., M.Z., S.C., Z.S., Ka.Ka, K.W., Marz,W., K.I., genetic testing; E.B., M.N.-K., K.P., A.K., Kat,P., K.Py., A.S.-P., Ann.W., A.P., P.J. in vitro research; B.P.J., A.W., M.P., J.G.K., G.S., J.R., T.W., A.E., D.K., S.Gr., T.R, A.P., L.G., A.P., W.K., L.B., K.W., K.K., T.S., G.B., W.W.J., J.H. patient management; M.L., S.G., A.W. analysis and interpretation of clinical data; P.J., E.B. analysis and interpretation of the in vitro data; M.L., E.B., P.J., A.W., S.G., O.H. manuscript writing; G.J.R. and senior author O.H. and A.W. construction of manuscript and critical revision. All authors accepted the final version of the manuscript.

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**Competing interests**
The authors declare no competing interests.

**Additional information**

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