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RNA expression of genes involved in cytarabine metabolism and transport predicts cytarabine response in acute myeloid leukemia

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Abstract

Background—Variation in terms of outcome and toxic side effects of treatment exists among acute myeloid leukemia (AML) patients on chemotherapy with cytarabine (Ara-C) and daunorubicin (Dnr). Candidate Ara-C metabolizing gene expression in primary AML cells is proposed to account for this variation.

Methods— *Ex vivo* Ara-C sensitivity was determined in primary AML samples using MTT assay. mRNA expression of candidate Ara-C metabolizing genes were evaluated by RQPCR analysis. Global gene expression profiling was carried out for identifying differentially expressed genes between *ex vivo* Ara-C sensitive and resistant samples.

Results—Wide interindividual variations in *ex vivo* Ara-C cytotoxicity were observed among samples from patients with AML and were stratified into sensitive, intermediately sensitive and resistant, based on IC50 values obtained by MTT assay. RNA expression of deoxycytidine kinase (DCK), human equilibrative nucleoside transporter-1 (ENT1) and ribonucleotide reductase M1 (RRM1) were significantly higher and cytidine deaminase (CDA) was significantly lower in *ex vivo* Ara-C sensitive samples. Higher DCK and RRM1 expression in AML patient's blast correlated with better DFS. Ara-C resistance index (RI), a mathematically derived quotient was proposed based on candidate gene expression pattern. Ara-C *ex vivo* sensitive samples were found to have significantly lower RI compared with resistant as well as samples from patients presenting with relapse. Patients with low RI supposedly highly sensitive to Ara-C were found to have higher incidence of induction death (p = 0.002; RR: 4.35 [95% CI: 1.69–11.22]). Global gene expression

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Ethical conduct of research
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profiling undertaken to find out additional contributors of Ara-C resistance identified many apoptosis as well as metabolic pathway genes to be differentially expressed between Ara-C resistant and sensitive samples.

Conclusion—This study highlights the importance of evaluating expression of candidate Ara-C metabolizing genes in predicting *ex vivo* drug response as well as treatment outcome. RI could be a predictor of *ex vivo* Ara-C response irrespective of cytogenetic and molecular risk groups and a potential biomarker for AML treatment outcome and toxicity.

Keywords

acute myeloid leukemia; cytarabine; drug resistance

Despite significant advances in the treatment of acute myeloid leukemia (AML), resistance, relapse and toxicities to the anticancer drugs remain a major hindrance to successful treatment outcome. The heterogeneous nature of AML both in terms of biology and treatment outcome highlights the importance of pretreatment risk stratification to distinguish patients who may get benefited from the therapy. The standard of care for AML over four decades has been the induction chemotherapy using a combination of cytarabine (Ara-C) and daunorubicin. A complete remission (CR) rate of 70–75% in younger adults (age less than 60) is documented after one or two cycles of induction with cytarabine and daunorubicin, but durable remissions are achieved only in 30–40% of patients. Approximately 50% of older patients are also documented to achieve remission, but unfortunately, 85% of those in remission will subsequently relapse [1,2].

The prerequisite for the cytotoxic action of prodrug Ara-C is the enzymatic conversion to its active triphosphorylated form, Ara-CTP. The amount of Ara-CTP formed determines the extent of cytotoxicity and treatment response [3,4]. Many drug activating (deoxycytidine kinase [*DCK*] and human equilibrative nucleoside transporter 1 [*ENT1*]) and deactivating genes (cytidine deaminase [*CDA*] and 5'nucleotidase [*NT5C2*]) which are involved in the transport and bio-transformation of cytarabine contribute to the variation in Ara-C sensitivity in AML patients (Figure 1).

Human ENT1 transports Ara-C into the cell, and its expression enhances the availability of the drug inside the cell. Previous studies have shown that decreased expression of ENT1 correlates with a decreased response to Ara-C [5,6]. DCK is the rate limiting kinase involved in the phosphorylation of Ara-C to Ara-CTP. The decreased expression and activity of DCK in AML has been reported as a mechanism of clinical resistance to Ara-C [7,8]. CDA brings about an increased systemic degradation of Ara-C by deaminating the compound to its uracil derivative, uracil arabinoside (Ara-U) [9]. Increased activity and expression of CDA was shown in AML patients who are nonresponders to induction chemotherapy when compared with those who respond well to Ara-C [10,11]. On the other hand, low CDA activity and expression has shown associated with severe drug-related toxicities [12,13]. NT5C2 opposes DCK activity by dephosphorylating the monophosphorylated form of drug back to Ara-C. Increased expression of cytosolic NT5C2 (also known as cN-II), mRNA in leukemic blasts at diagnosis has been shown associated with a higher relapse rate and shorter disease-free survival (DFS) [7,14]. *De novo* nucleotide bio-synthesis pathway is shown to cross talk with

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Ara-C activation pathway through ribonucleotide reductase (RR). Increased RR activity is shown to increase the *de novo* dCTP pools and is speculated to competitively decrease the binding of Ara-CTP to DNA resulting in reduced cytotoxicity. The RR holoenzyme is a dimeric protein and comprises of large and small subunits, ribonucleotide reductase M1 (RRM1) and ribonucleotide reductase M2 (RRM2). In patients with advanced non-small-cell lung cancer (NSCLC) treated with gemcitabine, a drug with similar metabolic activation, low RRM1 mRNA expression has shown associated with significantly longer median survival [15].

Technological advances in molecular genetics have helped in identifying genetic markers which prognosticate and predict treatment outcome in AML. In addition to the core -binding factor (CBF), leukemias with translocations t(8:21)(q22;q22) or inv(16) (p13q22)/t(16;16) (p13;q22) that have been associated with good prognosis in patients with AML receiving conventional chemotherapy, mutation status of genes encoding nucleophosmin (NPM1), Fms-like tyrosine kinase 3 (FLT3) and CCAAT/enhancer binding protein alpha (CEBPA) have also been shown to have prognostic relevance in AML with normal karyotype (NK-AML). Overexpression of Wilm's tumor-1 (WT1), brain and acute leukemia, cytoplasmic (BAALC), ETS-related gene-1 (ERG1), FLT3 and Meningioma-1 (MN1) RNA has also been shown to be associated with poor outcome in AML [16–18]. Identifying markers associated with drug-associated toxicities are also equally relevant as the therapeutic window for nucleoside analogs like Ara-C is very narrow and interindividual variability in the drug metabolizing genes could lead to life-threatening toxicities and treatment failure.

Few reports in the past have shown drug-metabolizing enzymes and transporters as important determinants of survival and outcome in AML [7,19]. We hypothesize that the RNA expression of drug-metabolizing enzymes and transporters are independent determinants of *ex vivo* as well as *in vivo* response to Ara-C in primary and relapsed AML, irrespective of molecular and cytogenetic markers. The present study aims to determine independent as well as the combined effect of mRNA expression of Ara-C metabolizing genes on Ara-C cytotoxicity and the role of cytogenetic and molecular markers on the mRNA expression of these genes in patients with AML at diagnosis.

Methods

Patients

A total of 260 adult patients with *de novo* AML (excluding AML-M3) at diagnosis before the initiation of therapy and 34 patients who presented with relapse between June 2009 and December 2013 at the Department of Hematology, Christian Medical College, Vellore, were enrolled in this study. Ten of these patients had neither RNA expression data nor *ex vivo* cytotoxicity data and were excluded from further analysis. Bone marrow samples were collected after obtaining written informed consent from the patients and this study was approved by the Institutional Review Board.

AML was subtyped according to WHO classification and cytogenetic risk stratification [20]. Immunophenotype by flow cytometry and bone marrow cytogenetic analysis of samples was performed in all patients at diagnosis and/or at relapse as a standard practice [21].

Treatment protocol

All patients who have undergone treatment received a conventional induction chemotherapy consisting of cytosine arabinoside administered as a continuous infusion at a dose of 200 mg/m^2 per day for 7 days along with 3 days of daunorubicin administered at a dose of 60 mg/m^2 per day for 3 days. Remission was documented using conventional definitions [22,23]. Induction failures include those patients who had persistent disease after at least two cycles of chemotherapy. Therapy for patients failing to achieve remission was individualized (done at treating physicians' discretion, which included intensive chemotherapy such as FLAG-Ida, hypomethylating agent therapy, allogeneic stem cell transplant and in some cases palliative care). Those achieving remission and having a HLA-matched sibling were offered an allogeneic stem cell transplant in CR1 with the exception of patients who had good risk cytogenetics. In patients achieving remission and not proceeding with an allogeneic stem cell transplant, three cycles of high-dose cytarabine consolidation (3 g/m^2 twice daily on alternative days for 3 days) were administered.

Ex vivo cytotoxicity assay

Ex vivo cytotoxicity of Ara-C was determined using the MTT assay, as described previously [24]. Briefly, bone marrow mononuclear cells (BMMNCs) were isolated using Ficoll-paque (GE Healthcare, CA, USA) and 1 × 10^5 cells were cultured in flat-bottomed 96-well microtiter plates in the presence of increasing concentrations of Ara-C (cytosine β-D-arabinofuranoside hydrochloride (Ara-C HCl; Sigma-Aldrich, MO, USA), ranging from 0.1 to 80.0 μM for 48 h. IC50 values were calculated using ADAPT 5 software [25]. Cells without drugs were used as untreated control and culture medium alone as reagent blank.

In parallel with IC50, area under the survival curve (AUC) was also calculated for all samples using the trapezoidal rule as reported previously [26]. The percent survival values at each concentration of Ara-C were determined after 48 h of exposure and plotted against Ara-C concentrations to generate a survival curve. Low AUC corresponds to Ara-C sensitivity as *ex vivo* cell kill is achieved within a small range of drug concentrations. IC50 and AUC correlated each other significantly (Supplementary Figure 1; see online at: www.futuremedicine.com/doi/suppl/10.2217/pgs.15.44).

Quantitative real-time PCR for drug-metabolizing genes

Total RNA was extracted from BMMNCs of AML samples using Tri Reagent (Sigma). Complementary DNA (cDNA) synthesis was performed with 1 μg RNA using Superscript II reverse transcriptase (Invitrogen, CA, USA) following the manufacturer's instructions. All RNA samples included in the study have undergone stringent multiple quality check assessment at various stages of experiment (RNA extraction, cDNA synthesis and RQPCR analysis). Concentration of the extracted RNA was determined by NanoDrop® ND-1000 UV, which measures the absorbance at 260 nm. RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an A260/A280 of 2.1, A260/A280 value of 1.8–2.1 was acceptable and taken for further experiments. RNA quality was assessed in all samples by running RNA samples in 1% nondenaturing agarose gel in TAE buffer. RNA should appear as two bright discrete bands that represent the 28S and 18S ribosomal species. The 28S band

should be brighter than the 18S band. Tailing of these major bands, or a background smear behind these bands indicate degradation of the RNA. Samples failing quality check are discarded and not considered for further cDNA synthesis.

TaqMan® Gene Expression Assays Hs 01040725_m1, Hs 00156401_m1, Hs 01085702_g1, Hs 01056737_g1 and Hs 01040690_g1 were used for detecting *DCK, CDA, ENT1, NT5C2* and *RRM1* mRNA expression, respectively, and were normalized to the house keeping gene *GAPDH* (Taqman assay ID 4352934E). Those samples whose GAPDH expression were low (CT > 25; indicative of poor cDNA quality), were excluded. RNA expression of Ara-C metabolizing genes were determined using ΔΔCT method, where the difference in the threshold cycle (ΔCT) values of target gene and the housekeeping gene *GAPDH* for each sample was normalized to ΔCT value of the sample AML001. We have alternatively used Abelson murine leukemia viral oncogene homolog 1 (ABL1) gene also as housekeeping gene, but *GAPDH* was found to be a better in context of Ara-C metabolizing genes, so was chosen as the housekeeping gene for normalizing gene expression in all samples.

Analysis of molecular markers

FLT3-ITD and NPM1 mutation status at diagnosis or/and at relapse were determined by Gene Scan analysis using genomic DNA samples as reported previously [27]. FLT3-TKD (D835) mutation was detected by PCR and restriction fragment length polymorphism analysis using EcoRV restriction enzyme (Fermentas, Ontario, Canada) [28]. Type of NPM1 mutation was determined by PCR followed by DNA sequencing using ABI 3130 genetic analyzer. RUNX1-RUNX1T1 and CBFB-MYH11 fusion transcripts were screened by RT-PCR [29].

Microarray analysis

Based on *ex vivo* Ara-C cytotoxicity at diagnosis, five Ara-C sensitive (IC50 < 6 μM AraC; median IC50: 1.8 μM [0.5–2.96]) and five Ara-C resistant samples (IC50 > 80 μM) were included for microarray analysis. The table (Supplementary Table 1) shows the Karyotype and NPM1/FLT3 mutation status of the samples taken for microarray analysis.

BMMNCs were isolated and total RNA was extracted. RNA samples were stored under -80 °C conditions until shipped to the microarray facility (Genotypic Technology, Bengaluru, India). Gene expression was assessed with Agilent Human Whole Genome 8 × 60k Microarray. Data were analyzed using GeneSpring GX Version 12.0 software from Agilent.

Statistical analysis

Comparisons between candidate gene expression with *ex vivo* cytotoxicity and molecular markers were performed using Kruskal–Wallis/Mann–Whitney U-test/Chi-squared test as applicable. All statistical tests were done using GraphPad Prism software. Multivariate analysis by logistic regression with covariates added to the regression in a stepwise fashion was also carried out to see the effect of candidate genes on ara-C *ex vivo* toxicity. The probability of survival was estimated with the use of the product-limit method of Kaplan-Meier for event-free survival (EFS) and DFS was compared using the log rank test [23]. EFS is determined from the date of diagnosis to relapse from CR, or death from any cause. DFS

(same as remission duration) is defined only for patients who achieve CR, and is measured from the date of attaining the leukemia-free state until the date of AML relapse [23]. All survival estimates are reported as ±1 SE. All p-values were two-sided, with values of 0.05 or less indicating statistical significance. Statistical analysis was done using the SPSS 16.0 Software (Chicago, USA).

Results

Patients

There were 156 males and 94 females in the *de novo* AML cohort with a median age of 42.5 years (range: 15–78 years) (Table 1). Among the 34 patients presented at relapse, 14 were females and 20 males, with median age of 33.5 years (16–67 years). Results of the molecular markers evaluated in these patients are listed (Table 1).

114 of the total patients enrolled in the study underwent conventional induction chemotherapy consisting of cytosine arabinoside and daunorubicin in our center and the demographics is as shown in Supplementary Table 2. The frequencies of cytogenetic and molecular markers of the treated group were comparable with the total patient cohort. In a self-paying system like ours, around 70% of the patients who are diagnosed with AML cannot afford treatment owing to financial constraints [30]. So in this study cohort, even though 250 *de novo* AML patients were enrolled only 114 had undergone treatment in our center and the rest received palliation or no treatment at all.

Wide variation exist in *ex vivo* cytotoxicity among AML patients

Of the 250 patients, only 188 had the Ara-C *ex vivo* cytotoxicity values due to inadequate cells available in the remaining 62 patients. The *ex vivo* cytotoxicity to Ara-C in AML patients exhibited a wide interindividual variation (median: Ara-C IC50: 6 μM [range: 0.24–79 μM]). Thirty seven of these patient's cells did not show 50% cell kill within the range of Ara-C concentration used in the assay and hence the IC50 was arbitrarily fixed as 80 μM, which was the maximum concentration of Ara-C used in the MTT assay. Based on IC50 values, the samples were stratified into three groups – sensitive, intermediately sensitive and resistant to Ara-C; Ara-C 'sensitive' group had the IC50 values below median 6 μM (n = 76), and those with above 6 μM were considered as 'intermediately sensitive' (n = 75) and the last group which did not show 50% cell kill in the assay (IC50 > 80 μM; n = 37) constituted the 'resistant' group.

AUC values were generated for all patients, even in the >80 uM group. The median AUC was determined to be 3055 (498–8119). The patients were stratified as AUC low (sensitive) and AUC high (resistant) based on median Ara-C AUC.

AML samples with higher mRNA expression of *DCK, ENT1* & *RRM1* & lower CDA expression were found to be sensitive to Ara-C by *ex vivo* cytotoxicity assay

We observed a wide variation in the mRNA expression pattern of candidate Ara-C metabolizing genes in AML patients (Supplementary Figure 2). Of these genes, *CDA*

showed maximum variation in AML patients (median expression: 95.64 [range: 0.1630–2760]) (Supplementary Figure 2).

The association of candidate Ara-C metabolizing gene expression with *ex vivo* Ara-C cytotoxicity was analyzed. RNA expression of *DCK* and *ENT1* were found to be significantly higher in Ara-C sensitive samples compared with those with intermediate sensitivity and Ara-C resistant; median *DCK* expression was 297.5 (61.56–1232) in sensitive samples compared with 213.4 (31.87–629.5) and 185.9 (48.68–1178) in intermediately sensitive and resistant samples, respectively ($p = 0.0006$). Similarly, *ENT1* expression was 190.5 (44.12–657.6) in sensitive when compared with 149.4 (21.55–916.2) and 130.6 (36.56–911.1) in intermediate and resistant samples ($p = 0.01$) (Figure 2A & B).

mRNA expression of CDA, the major Ara-C deactivating gene was significantly lower in Ara-C sensitive samples (median expression: 43.1 [0.46–1467]) as compared with intermediately sensitive and resistant groups, respectively, (127.3 [0.16–2760] and 111.1 [0.239–2150]; $p = 0.02$; Figure 2C).

RRM1 expression was significantly higher in Ara-C sensitive samples (median expression: 169.6 [20.66–878.7]) when compared with intermediate (108.1 [26.65–851.2]) and resistant groups (131.6 [29–627.1]; $p = 0.03$; Figure 2D), while *NT5C2* expression did not show any significant association with *ex vivo* Ara-C cytotoxicity. A similar association was observed, when these genes were evaluated with AUC (Supplementary Figure 3). Multivariate analysis shows that these candidate genes are significantly associated with *ex vivo* toxicity ($p = 0.0029$).

Resistance index could be predictive of Ara-C sensitivity

Based on the above findings, we proposed Ara-C resistance index (RI). RI is calculated by the formula:

$$RI = \Delta CT\ (DCK \times ENT1)/\Delta CT\ CDA.$$

RI is a mathematically derived quotient obtained by dividing the products of ΔCTs of Ara-C activating genes *DCK* and *ENT* by ΔCT of the Ara-C deactivating gene *CDA.*

RI values were significantly higher in resistant (IC50 > 80 μM) and intermediately sensitive (IC50 > 6 μM) compared with sensitive samples (median: 5.32 [1.759–11.82]) and 5.27 [1.89–11.62] vs 3.79 [1.59– 9.8]; $p < 0.0001$; Figure 3A). A similar analysis with AUC of Ara-C and RI was also done; median RI was found to be higher in samples with high AUC values (RI: 5.28 [1.76–11.82] vs RI 4.08 [1.59–10.4]; $p = 0.0002$; Figure 3B).

RI was also determined for patients who presented at relapse ($n = 34$), though there were no paired diagnostic samples available for this cohort. The median RI in the relapse cohort was significantly higher (RI: 5.36 [2.01–19.85]) compared with that of sensitive group (RI: 3.79 [1.59–9.8]; $p = 0.0001$; Figure 3C). Intermediately sensitive and resistant samples were shown to have comparable RI values with that of relapse patients. Similarly, low AUC patients also displayed much lower RI values (4.08 [1.59–10.4]) when compared with the relapse cohort ($p = 0.001$)

Increased incidence of induction death in patients with high RI

Since the *ex vivo* data suggested RI to be a strong predictor of Ara-C sensitivity, the clinical significance of RI was evaluated in 114 patients who had undergone treatment in our center. Surprisingly, patients with low RI (RI < 3.21; 25th percentile and lower) had significantly poorer EFS (p = 0.013; Figure 4A). Further analysis revealed that the patients with low RI, who supposedly to be very sensitive to Ara-C *ex vivo,* had increased incidence of induction death (46% of patients in the low RI group had induction death (p = 0.001; $\chi^2 = 9.96$; Figure 4B). Cox regression analysis also identified patients with low RI to have significantly higher risk toward induction death (p = 0.002; RR: 4.35 (95% CI: 1.69–11.22). There was no significant association with RI and DFS.

Ara-C pathway gene expression predicts DFS

Patients having RRM1 expression in the lowest quartile had a significantly shorter DFS when compared with the rest (p = 0.03; Figure 4C). Patients with high DCK (expression greater than median, 243.1) were found to have a better DFS, though not reaching statistical significance (p = 0.21; Figure 4D). This could probably be due to increased incidence of relapses occurring in patients with low DCK and RRM1.

Differentially expressed genes between *ex vivo* Ara-C sensitive & Ara-C resistant samples by global gene expression profiling

Using unpaired t-Test, 4436 genes were identified to be differentially expressed (fold-change expression values were provided as log-base 2) between Ara-C sensitive samples and Ara-C resistant samples (Supplementary Figure 4). The differentially expressed genes fell into the following biological processes such as transcription (375 genes), transport (364 genes), metabolism (267 genes; Figure 5A), immune (155 genes), cell cycle (129 genes) and apoptosis (123 gene; Figure 5B). Upregulated gene list in Ara-C sensitive group included apoptotic-related genes like *PMAIP1*, *NDUFA*, *BAX*, *BCL2* and *TRAF2*. Transcriptional regulators including *SMAD5*, *SMAD1*, ZNF family proteins- *ZNF644*, *ZNF469*, *ZNF195*, *ZNF22*, *ZNF3*, *ZNF713*, *ZNF777*, *ZNF234*, *CEBPα*, *PARP*, *ETV6*, *E2F3* were also shown to be upregulated. Downregulated genes in Ara-C sensitive group are of special interest as they could be potential candidates for targeting Ara-C nonresponsive group. They included transcriptional regulators like *HDAC4*, *KLF4*, *CREB5*, *CEBPβ*, *RARA*, *E2F4*, *MNDA*, *MTA3* and *PPARγ* and also apoptotic genes like *MCL1*, *PSEN1*, *ELMO2*, *PAK1*, *APAF1*, *MAPK1*, *CD40*, *FAS*, *CASP1* and *CASP8*. Interestingly, many of the genes involved in cellular metabolism were found to be downregulated in Ara-C sensitive group. Major downregulated metabolic genes included CDA, *SLC2A3*, *SLC2A8*, *GK*, *NADK*, *ACACB*, *ACSL1*, *PFKFB4*, *PFKFB3*, *PDK4*, *ME1* and *PC*.

Fifteen genes under the broad functional categories – Apoptosis and Metabolism related were evaluated in 28 samples comprising of 15 *ex vivo* Ara-C sensitive and 13 resistant samples (based on Ara-C IC50 < 6 and > 80 uM). The genes evaluated under metabolism-related category included *ACSL1*, *PPARγ*, *RXRα*, *SLC2A3*, *SLC11A1*, *ME1* and *HK3*. The genes under apoptosis category included *CEBPβ*, *PMAIP1*, *PAK* and *CNOT7*. The subunits of mitochondrial respiratory chain NADH dehydrogenase (complex1)-*NDUFA1* and *NDUFA13* and mitochondrial ATP synthase complex subunits (*ATP5L* and *ATP5I*) were

also evaluated. When validated by RQ-PCR, all the genes screened showed results consistent with the microarray data as shown in Supplementary Figure 5A–O. The mitochondrial respiratory chain genes *ATP5L* and *ATP5I* and *NDUFA13* (Supplementary Figure 5A–C) were found overexpressed in the Ara-C sensitive samples. The metabolism-related genes *SLC2A3* (glucose transporter, Supplementary Figure 5E), *SLC11A1* (divalent metal ion transporter), *ACSL1* (acyl-CoA synthetase, Supplementary Figure 5F), *HK3* (hexokinase) and *ME1* (malic enzyme), *PPARγ* and *RXRα* were found to be overexpressed in Ara-C resistant samples and are consistent with the data obtained from the microarray. Apoptosis-related genes *PAK1 and CEBPβ* were found overexpressed though not statistically significant (p = 0.1 and 0.2, respectively) in Ara-C resistant samples, whereas *CNOT7* (Supplementary Figure 5D) and *PMAIP1* (NOXA) were higher in Ara-C sensitive samples.

Discussion

AML being a heterogeneous disease in terms of biology and treatment outcome highlights the importance of pretreatment risk stratification to distinguish patients who may get benefited from therapy. Cytogenetics and age at diagnosis are among the most acceptable predictors for treatment response, relapse risk and over-all survival. Previous studies have shown that altered expression of nucleotide salvage pathway genes may contribute to the resistance to nucleoside analogs like cytarabine and gemcitabine [8,31]. In this study, we have analyzed the role of expression of the major genes involved in cytarabine transport and metabolism on *ex vivo* as well as the clinical Ara-C response and compared it with cytogenetic and molecular markers in AML. Our candidate gene expression data led us to propose Ara-C RI, which is shown to be associated with both *ex vivo* as well as *in vivo* Ara-C responses.

The biochemical basis of Ara-C resistance was attributed to decrease in the amount of Ara-C activating enzyme DCK, as early as 1970 [32]. DCK activity and expression had been shown to be associated with better treatment outcome when treated with nucleoside analogs such as Ara-C, gemcitabine, cladribine, fludarabine and clofarabine [7,33–35]. Galmarini *et al.* in their study have shown that when treated with Ara-C, patients with higher *DCK* expression demonstrated longer EFS than patients with low DCK expression [7]. In our study, patients with high DCK levels had better *ex vivo* sensitivity toward Ara-C and also associated with better DFS though not reaching statistical significance, probably owing to smaller number of treated patients. Similarly, few previous studies have indicated *ENT1* expression to be directly correlated with the drug response toward Ara-C, so as with other nucleotide analogs [6,36,37]. Our gene expression analysis also showed a similar positive correlation with *ex vivo* Ara-C sensitivity where higher mRNA levels of *ENT1* were observed in drug-sensitive patients.

Increased expression of *CDA* was shown to be a negative prognostic factor in AML patients treated with Ara-C-containing regimens, presumably due to increased systemic deamination of Ara-C to the noncytotoxic Ara-U [38]. CDA enzyme/RNA levels were also shown to influence gemcitabine and Ara-C cytotoxicity and development of drug-related toxicity due to defective clearance of the drug [39,40]. Among all the candidate genes, *CDA* expression showed maximum fold variation in this study (Supplementary Figure 2). This variation in

expression could be attributed to the highly polymorphic nature of *CDA* gene [24], and many of these polymorphisms have been shown to influence treatment outcome [40,41]. *CDA* expression was found to be significantly lower in patients who were sensitive to Ara-C when compared with those resistant *ex vivo* (IC50 > 6 μM). *NT5C2* expression and genetic variants have also shown associated with the development of resistance to clinically important nucleoside analogs like Ara-C and gemcitabine [42,43]. NT5C2 activity opposes that of DCK by dephosphorylating Ara-CMP, thereby decreasing the production of the active triphosphate form, Ara-CTP resulting in decreased disease-free and overall survival in adult AML patients undergoing treatment with Ara-C [14]. The deactivating role of *NT5C2* was not observed in our cohort of patients, with respect to *ex vivo* Ara-C cytotoxicity and RNA expression. It must not be overlooked that the inhibitory effect of NT5C2 is at the enzymatic level and not at the RNA level.

RRM1 overexpression has been shown to be associated with gemcitabine resistance in human pancreatic cancer and in NSCLC cell lines [44,45]. At the same time, NSCLC patients with high RRM1 expression who received non-gemcitabine based therapy have shown to have significantly longer overall survival and progression-free survival [46,47]. In the present study, patients with high RRM1 expression had better *ex vivo* as well as *in vivo* Ara-C response. There are no reports from AML where RRM1 expression is shown associated with survival, though a recent report have shown association of RRM1 genetic variants and treatment outcome [48]. It is speculated that RRM1 might not be a direct molecular target for Ara-C as that in case of gemcitabine, even though both the drugs have similar metabolic activation. The candidate Ara-C metabolic genes evaluated in this study were in the gene list associated with cytarabine pharmacogenomics in PharmGKB database [49]. This is a growing list and additional gene targets in PharmGKB could also have possible implications on cytarabine metabolic activation and on clinical outcome.

When we evaluated the effect of previously reported molecular and cytogenetic markers in AML on expression of individual Ara-C metabolizing genes, no significant association was found between any of these markers except NPM1. Mutations in *NPM1* gene are recognized as the most frequently occurring mutations in AML, especially in NK-AML. Unique gene signatures like overexpression of *HOX* and *MEIS* family genes have been associated with NPM1 mutations [50]; however, the mechanisms by which this mutation affect drug response still remains to be elucidated. Our data have shown for the first time that the good prognosis associated with the NPM1 mutation might be due to the overexpression of *DCK* in these patients (Supplementary Table 3). *In silico* analysis of *DCK* promoter using TF search [51] showed potential binding sites for *HOX* and *MEIS* family of transcription factors, which probably explains the increased *DCK* expression in NPM1-mutated AML. A previous report has shown FLT3-ITD mutation to repress *ENT1* expression thereby inducing Ara-C resistance [22]; however, we have not seen any such association in the present study.

Ara-C RI was found to be significantly associated with *ex vivo* Ara-C sensitivity where patients with higher RI values were found likely to have high IC50 as well as high AUC values. Patients who presented at relapse, also showed higher RI values similar to that of *ex vivo* resistant samples at diagnosis. However, when RI was evaluated in the clinical scenario, despite having higher Ara-C sensitivity in *ex vivo,* low RI patients were found to have

shorter EFS. Further evaluation of these patients revealed higher incidence of induction death in this group, which probably resulted in shorter EFS. Increased Ara-C sensitivity leading to drug-related toxicity is speculated to be the cause of induction death in such patients. This association could be of great relevance as induction death is still a major challenge in AML treatment particularly in developing countries. Further validation of this index in a large cohort of AML patients is warranted. Our recent report also supports this notion where patients with high DCK/CDA expression ratio are shown to be more susceptible to Ara-C-specific toxicities including cerebellar and pulmonary toxicities [13]. We did not find any significant association with RI and DFS, but we presume RI could have association with DFS in a more uniform cohort of AML patients, where induction death is not a major cause of treatment failure.

None of the molecular markers, including FLT3-ITD, FLT3-TKD, NPM1 mutation, RUNX1-RUNX1T1 and CBFB-MYH11 fusion transcripts and CD34 expression, showed any significant association with Ara-C RI or IC50. Though these markers have been shown to be of prognostic relevance in AML, the mechanism is not yet known. Lack of association of these prognostic markers with RI suggests that RI could be an independent marker of *ex vivo* Ara-C sensitivity (Supplementary Table 4).

Though expression of Ara-C metabolizing genes correlated well with *ex vivo* cytotoxicity as well as treatment response *in vivo,* we undertook a genome-wide gene expression profiling to address possible mechanisms of Ara-C resistance other than the candidate genes. There are no microarray data available in AML to our knowledge, where cytarabine *ex vivo* sensitive and resistant samples were compared. Of the differentially expressed gene families, genes involved in apoptosis and metabolism were studied with special interest. It was noted that in Ara-C sensitive samples, proapoptotic genes like *BAX*, *PMAIP1* (Noxa), *NDUFA13*, *DAPK3*, *CASP 3*, *CASP6* were upregulated, and at the same time antiapoptotic genes like *TEGT* (BAXI1), *MCL1*, *SERPINB2*, *NOD2*, *PAK1* were downregulated. Downregulated genes in Ara-C sensitive group are of special interest as they could be potential candidates for targeting Ara-C nonresponsive group, and also many of these genes are druggable targets. Interestingly, several genes involved in cellular metabolism were found to be downregulated in Ara-C sensitive group. Major downregulated metabolic genes include *SLC2A3*, *SLC2A8*, *GK*, *NADK*, *ACACB*, *ACSL1*, *PFKFB4*, *PFKFB3*, *PDK4*, *ME1* and *PC.* This study has come up with previously unrecognized aspects in Ara-C resistance in AML, and suggests that Ara-C sensitive samples have decreased expression of 'Warburg genes' and the 'antiapoptotic' genes. Our data as well as growing evidences from various malignancies and altered cellular metabolism propose the possibility of using metabolic inhibitors as well as antiapoptotic inhibitors alone or in combination with Ara-C to overcome drug resistance. AraC-RI is shown here as an independent predictor of *ex vivo* Ara-C efficacy and toxicity irrespective of cytogenetic and molecular risk groups. This report showing new insights about Ara-C response in AML patients could potentially form the basis for predicting clinical response in the future.

Future perspective

Ara-C RI which correlated significantly with *ex vivo* Ara-C sensitivity and increased treatment-related mortality must be explored in an independent cohort and could have major therapeutic implications. The microarray analysis has provided many important leads, which need to be addressed further. Many genes were identified to be differentially expressed, of which the apoptosis and metabolism-related genes are believed to have huge therapeutic potential mainly because of the 'druggability' of these pathways. While the current study addresses only resistance toward cytarabine, the role of combined daunorubicin and cytarabine resistance needs to be evaluated for completely deciphering AML chemotherapeutic resistance and is currently ongoing in the laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Financial & competing interests disclosure

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Executive summary

- Cytarabine (Ara-C) pathway gene expression correlated well with Ara-C *ex vivo* cytotoxicity as well as treatment response.
- Expression of *DCK, ENT1* and *RRM1* were found to be significantly higher in the *ex vivo* Ara-C sensitive samples and *CDA* expression was found to be significantly higher in *ex vivo* Ara-C resistant samples.
- The novel comprehensive index, cytarabine resistance index (Ara-C RI) correlated significantly with *ex vivo* Ara-C sensitivity and is proposed as a predictor of Ara-C sensitivity. RI is a mathematically derived quotient obtained by dividing the products of ΔCTs of Ara-C activating genes *DCK* and *ENT* by ΔCT of the Ara-C deactivating gene *CDA.*
- Patients presented with relapse were found to have significantly higher RI when compared with Ara-C sensitive samples at diagnosis.
- Patients with very low RI (in the lowest quartile) were found significantly associated with a greater risk of induction death. Higher Ara-C sensitivity leading to higher drug-related toxicity could be one main cause for induction death in such patients
- Our microarray analysis identified apoptotic as well as genes involved in cellular metabolism to be differentially expressed between Ara-C sensitive and Ara-C resistant samples and propose these may be additional signatures of Ara-C resistance.

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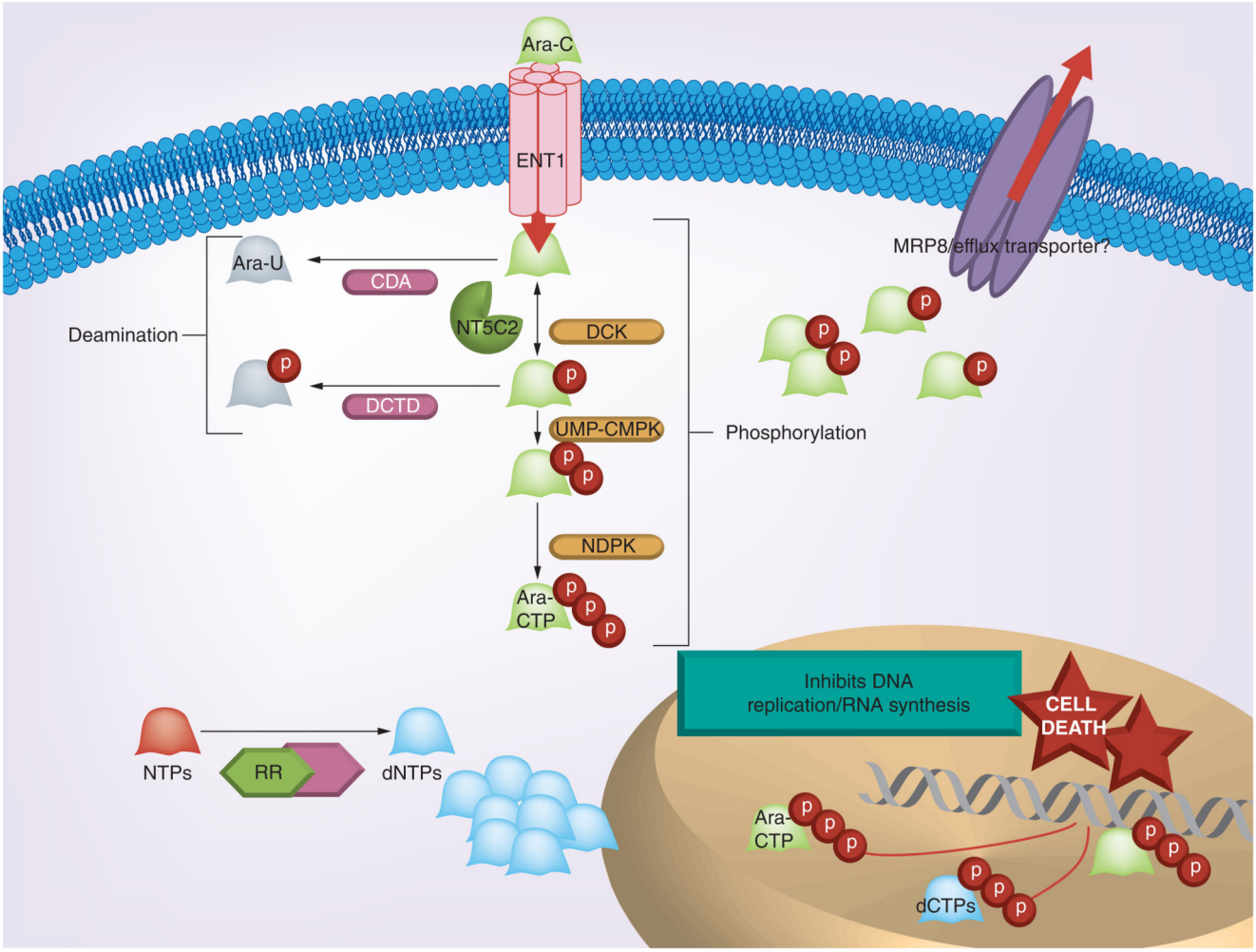

Figure 1. Metabolic pathway of Ara-C: key candidate genes involved in the metabolic activation of Ara-C are shown – ENT1 aids the transport of Ara-C into the cell.

Deoxycytidine kinase and the other activating kinases catalyze the conversion of inactive Ara-C to the active Ara-CTP. NT5C2, CDA and DCTD are the major deactivating enzymes. Ribonucleotide reductase increases the dNTP pool, which competitively inhibits the binding of Ara-CTP to DNA and thereby inhibits the cytotoxic action. MRP8 is an efflux transporter which effluxs out monophosphorylated nucleoside analogs.

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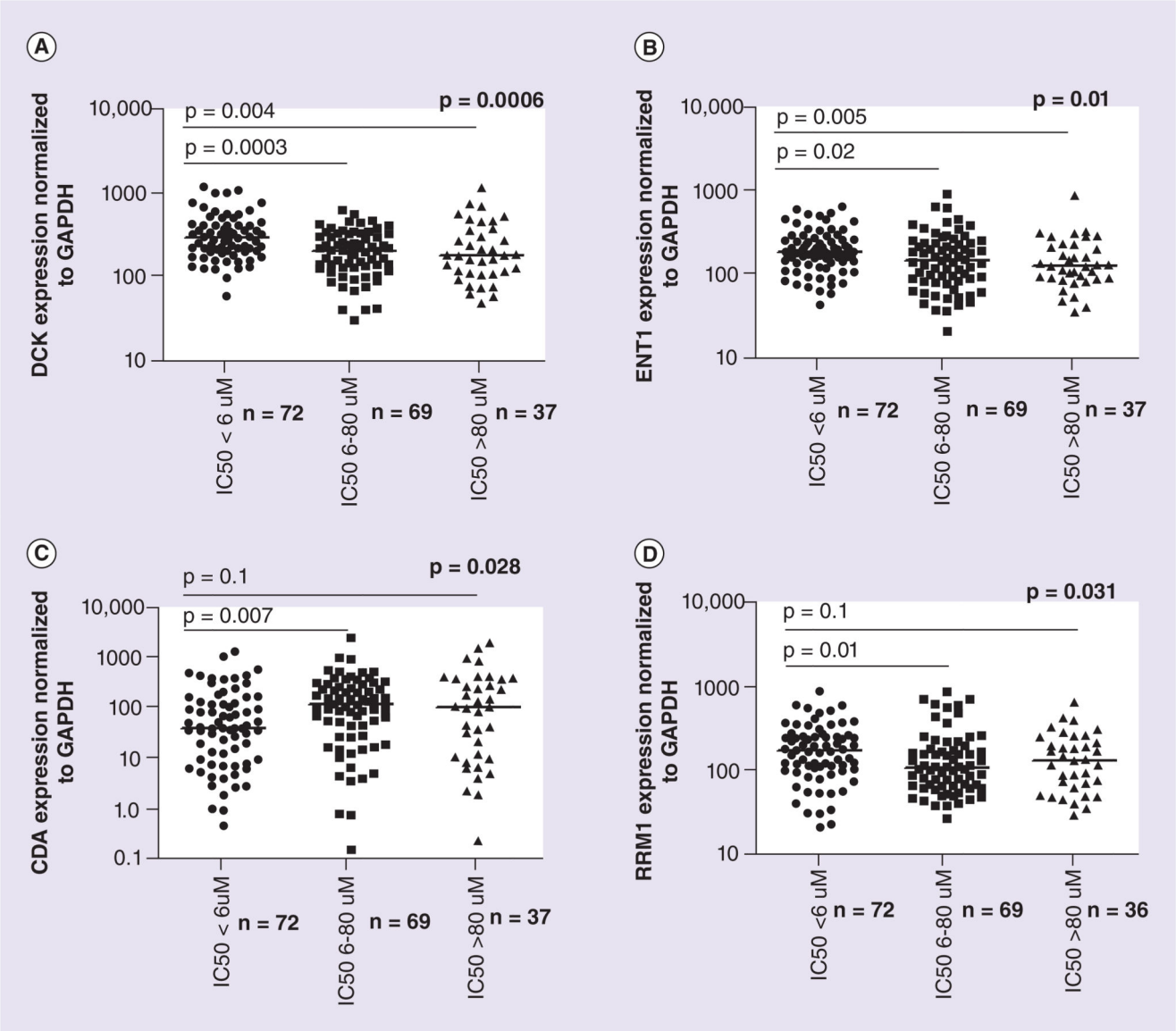

Figure 2. Ara-C metabolizing genes expression across different ex *vivo* cytotoxicity groups.

RNA expression of Ara-C metabolizing genes DCK, ENT1, CDA and RRM1 in different *ex vivo* cytotoxicity groups based on IC50: **(A)** *DCK,* **(B)** *ENT1,* **(C)** *CDA* and **(D)** *RRM1.* RNA expression for each gene was normalized to *GAPDH* and the relative expression was calculated in comparison to that of AML001. Lines represent median relative expression for each *p-value obtained by comparing the groups by ANOVA. Statistical significance was measured using the nonparametric Kruskal–Wallis test.

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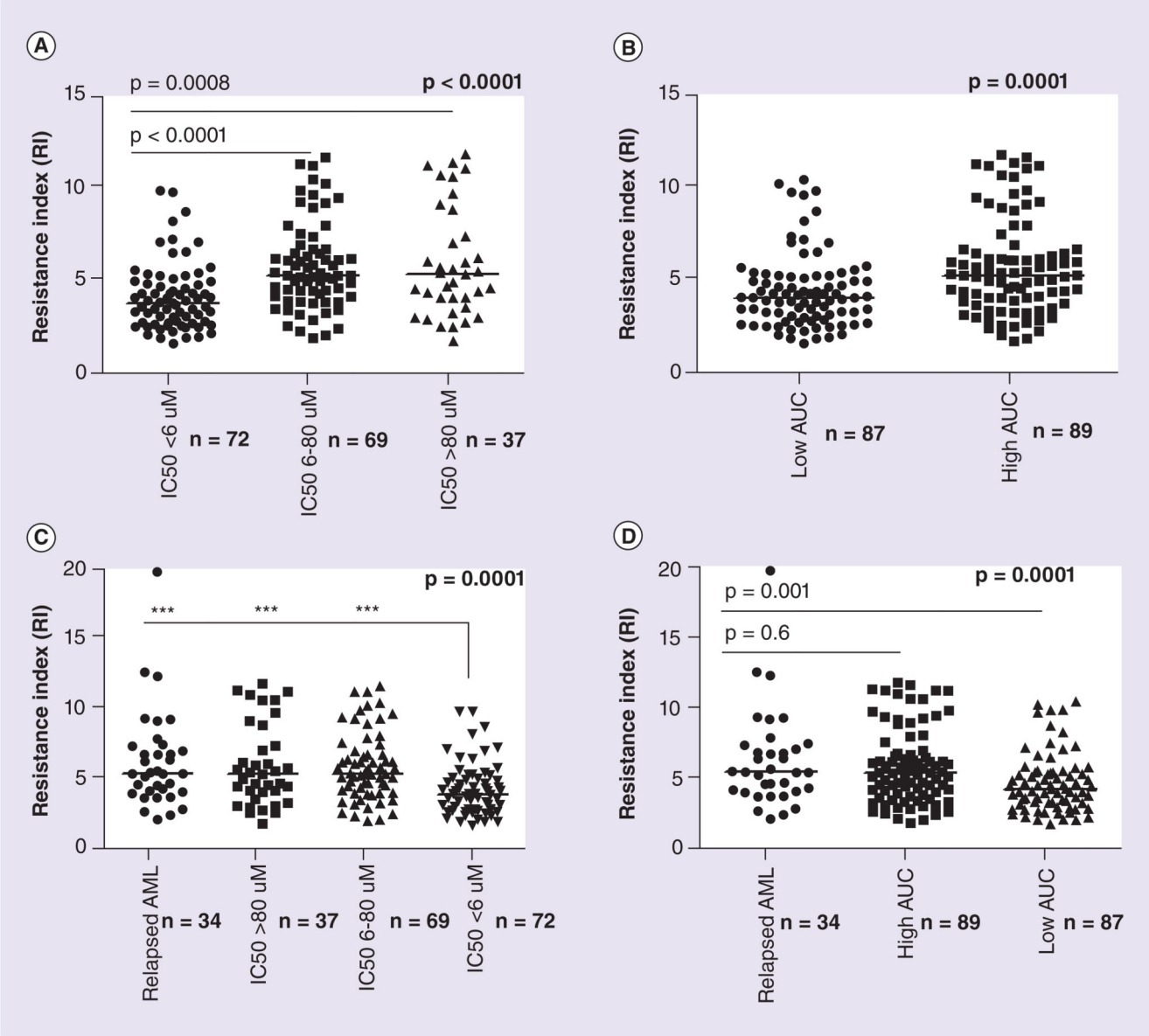

Figure 3. Ara-C resistance index across different *ex vivo* cytotoxicity groups and in relapsed acute myeloid leukemia.

Ara-C resistance index across different *ex vivo* cytotoxicity groups and in relapsed acute myeloid leukemia: **(A)** RI across different *ex vivo* cytotoxicity groups based on IC50 obtained from MTT assay. **(B)** RI in low AUC and high AUC groups. **(C)** RI in relapse patients compared with *ex vivo* resistant, intermediate and sensitive patients at diagnosis. **(D)** RI in relapse patients compared with low AUC and high AUC groups at diagnosis. Lines represent median values for RI in each group. Statistical significance was measured using nonparametric Kruskal–Wallis or Mann–Whitney test.

AUC: Area under the survival curve; RI: Resistance index.

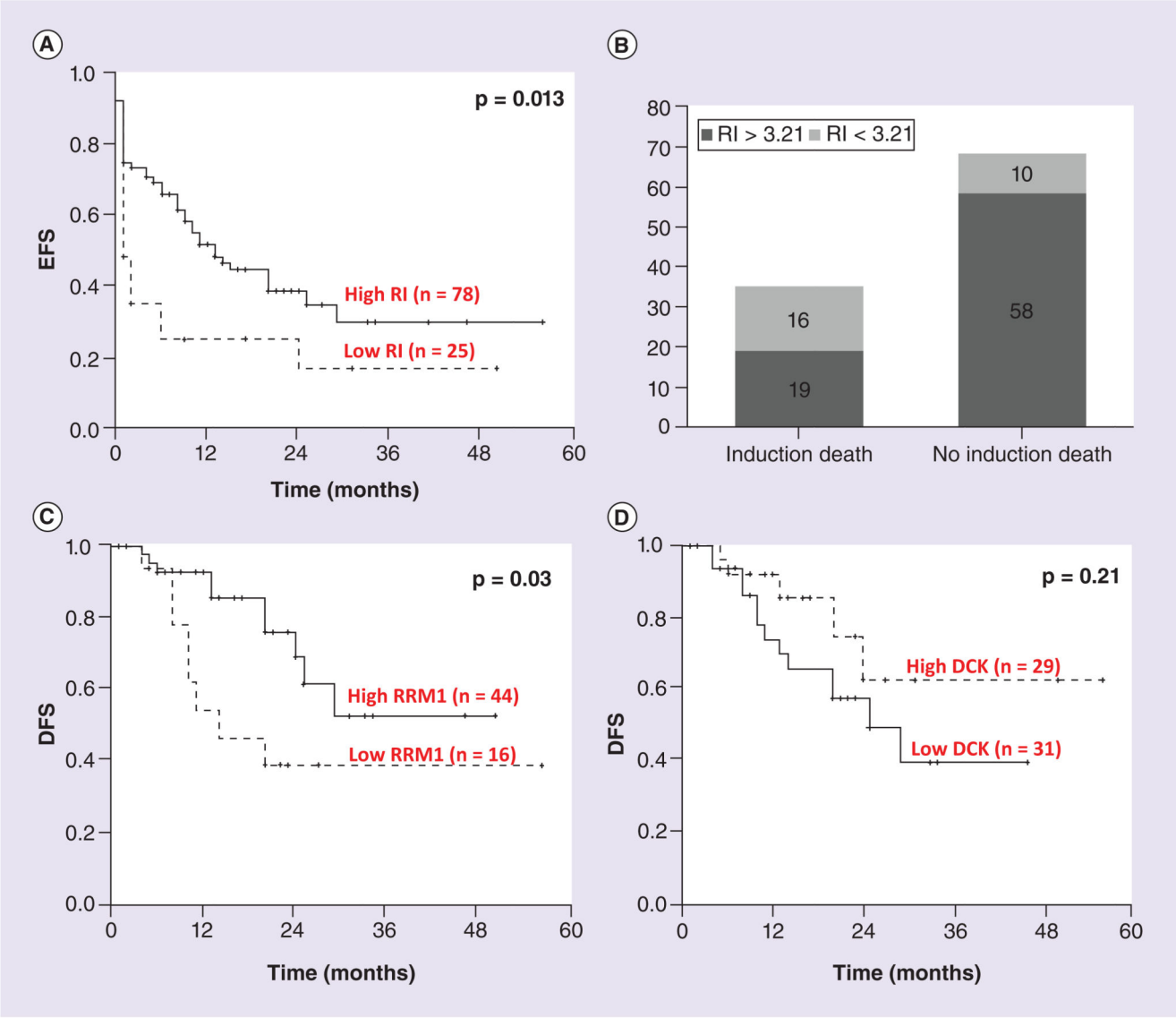

Figure 4. Kaplan–Meier survival estimates for RI, DCK and RRM1 expression.

(A) EFS comparison for patients with lowest quartile RI (RI < 3.21) versus the rest (RI > 3.21). **(B)** Bar graph showing the number of patients with low RI (RI < 3.21) and high RI (RI > 3.21) with or without induction death. **(C & D)** DFS comparison with respect to RRM1 (lowest quartile vs the rest) and DCK (based on median).DFS: Disease-free survival.

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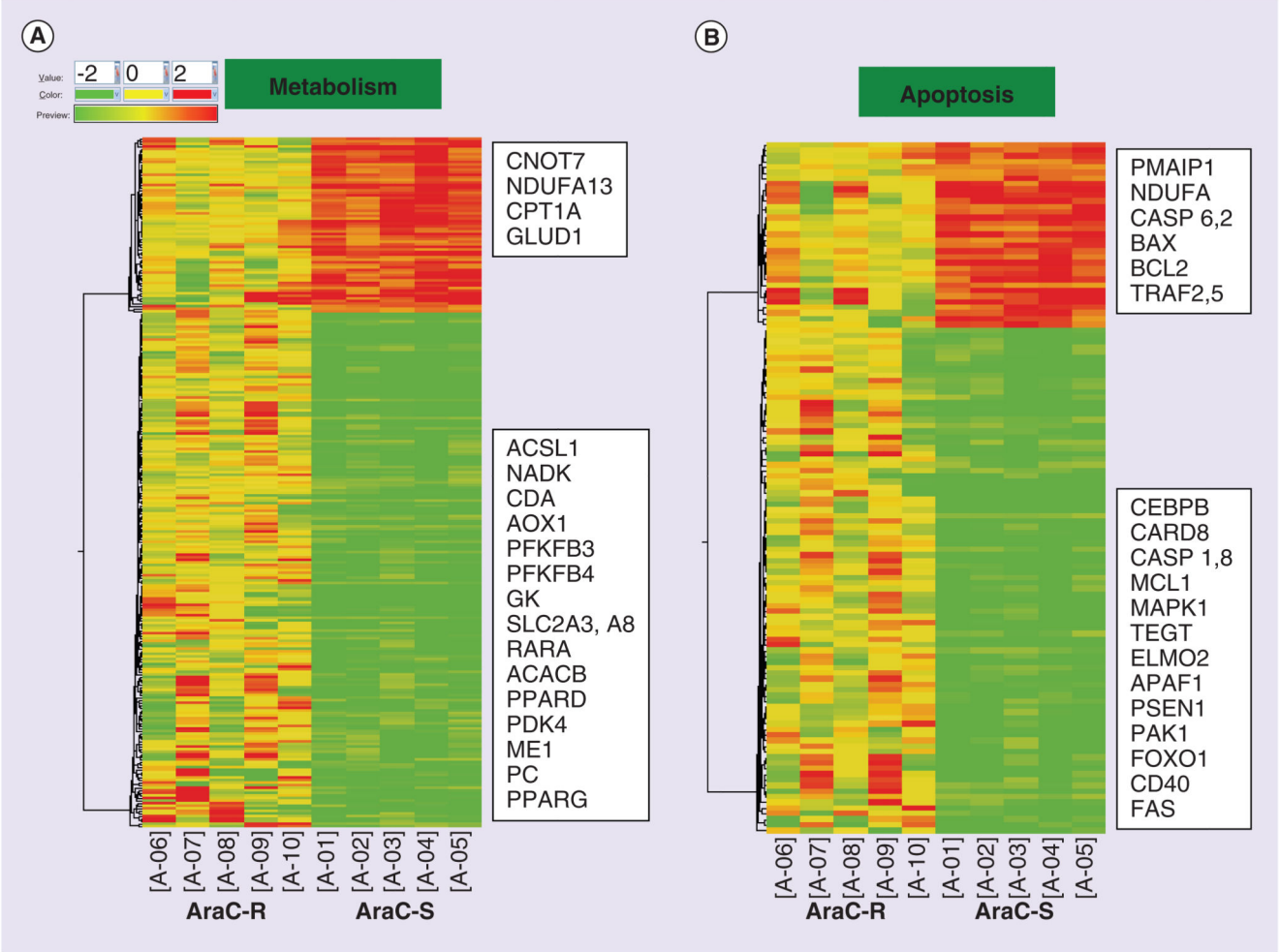

Figure 5. Heat maps showing differentially expressed genes using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant gene expression patterns.

Genes were classified based on functional category and pathways using GeneSpring GX. Here, differentially expressed genes under the functional categories metabolism **(A)** and apoptosis **(B)** are shown.

Table 1

Patient demographics. None of the patients in this cohort had myeloid sarcoma; secondary and therapy-related acute myeloid leukemia patients were not included. Cytogenetic data were not available for two patients.

Patient demographics	
***De novo* AML (n = 250)**	**Median (range)**
Age	42.5 years (15–78 years)
Sex	Males: 156, females: 94
Total WBC	15,400/cu mm (166–480,000)
Platelet count	32,000/cu mm (4000–486,000)
Hb	8.2 mg% (3–18)
Blast%	68% (21–100)
LDH	850 U/l (206–9963)
Creatinine	1 mg% (0.54–4.7)
WHO classification (n = 250)	
AML with recurrent genetic abnormalities	101 (40.4%)
AML with MDS-related features	44 (17.6%)
AML not otherwise specified	105 (42.0%)
Cytogenetic risk group (n = 248)	
Favorable	27 (10.9%)
Intermediate	144 (58.1%)
Adverse	77 (31.0%)
Normal karyotype	112 (45.1%)
Molecular markers	
NPM1 mutation (n = 245)	79 (32.3%)
FLT3-ITD (n = 245)	43 (17.55%)
FLT3-TKD (n = 244)	10 (4.3%)
t (8:21) (RUNX1-RUNX1T1) (n = 244)	20 (8.9%)
inv 16 (CBFB-MYH11) (n = 244)	11 (4.7%)