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ORIGINAL RESEARCH

Gene expression of *hENT1*, *dCK*, *CDA*, *dCMPD* and topoisomerase IIα as an indicator of chemotherapy response in AML treated with cytarabine and daunorubicin

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Purpose: Acute myeloid leukemia patients are commonly treated with cytarabine (Ara-C) and anthracyclines but the sustained remission rate is not very promising. We explored the role of drug-metabolizing enzymes and transporters in the therapeutic response.

Patients and methods: Bone marrow and peripheral blood samples of 90 newly diagnosed acute myeloid leukemia patients treated with standard 3+7 regimen were analyzed through real-time PCR for expression of human equilibrative nucleoside transporter 1, deoxycytidine kinase, cytidine deaminase (*CDA*), deoxycytidine monophosphate deaminase (*dCMPD*) and topoisomerase IIα (*Topo-IIa*). The expression of these markers was studied in relationship with good (persistent remission) and poor therapeutic response (relapse/resistance).

Results: High *Topo-IIa* expression in peripheral blood was associated with good response (P=0.006). Relapse was higher among low expressors of *Topo-IIa* in peripheral blood (OR: 26.25). Bone marrow *Topo-IIa* expression followed a similar trend but did not reach statistical significance. In contrast, patients with high bone marrow *dCMPD* expression had poor response (OR: 3; P=0.043). One-year disease-free survival (DFS) was better among those with high bone marrow *Topo-IIa* (P=0.04) or *CDA* (P=0.03) expression. High bone marrow *Topo-IIa* expression also had better DFS at 6 months (P=0.04) and at 12 months (P=0.04).

Conclusion: High expression of *Topo-IIa* in peripheral blood is a favorable indicator of persistent remission, good therapeutic response and DFS. High *dCMPD* and low *CDA* expression in bone marrow is associated with poor therapeutic outcome.

Keywords: topoisomerase IIα, *hENT1*, *dCMPD*, *CDA*, *dCK*, survival, AML, antimetabolites, anthracyclines

Introduction

Acute myeloid leukemia (AML) is mainly treated with chemotherapy regimen comprising of cytarabine and anthracyclines.[1] Treatment of AML is often complicated by resistance to conventional chemotherapy, which reflects the survival of tumor cells despite exposure to cytotoxic chemotherapeutic agents.[2,3] This has been a major problem in effective cancer treatment.[4] Therapeutic response depends on availability of drug at the target tissue where it inflicts damage to the malignant cells. Therefore, sensitivity and resistance to a drug could be influenced by the expression of drug transporters, drug metabolizing enzymes, and thus availability of sufficient drug at target receptors.

Human equilibrative nucleoside transporter 1 (*hENT1*) or solute carrier family 29 member 1 is a transmembrane glycoprotein present in cell and mitochondrial membranes. It is primarily involved in importing nucleosides for salvage pathway of

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purine and pyrimidine nucleosides. Cytotoxic nucleoside analogs, such as cytarabine (Ara-C), also utilize this transporter to enter cells.[5] Thus, efficiency of Ara-C influx can determine the intracellular drug level and thereby response to chemotherapy.[6]

Deoxycytidine kinase (*dCK*) is the rate-limiting enzyme of salvage pathway that phosphorylates nucleoside analogs, including Ara-C and gemcitabine to respective monophosphates. These monophosphates are phosphorylated by various kinases to diphosphates and triphosphates, which are in turn utilized for DNA synthesis. These Ara-C nucleotides inhibit DNA replication and transcription by inhibiting DNA and RNA polymerases.[7] Ara-C is converted to its inactive metabolite Ara-uridine through cytidine deaminase (*CDA*) while Ara-CMP is converted to an inactive metabolite, Ara-UMP by deoxycytidine monophosphate deaminase (*dCMPD*).[8] Both in vitro and in vivo studies have shown that changes in the cellular levels of *dCK*, *CDA* and *dCMPD* are associated with changes in intracellular drug concentrations. Thus, the efficacy of a specific dose of Ara-C may also vary depending upon expression of these enzymes as they may alter the availability of drug at the target.[9–12]

Topoisomerases, such as topoisomerase IIα (*Topo-IIa*), are enzymes required for relieving DNA supercoils during replication, transcription and chromosome condensation.[13] *Topo-IIa* expression starts increasing during S phase of cell cycle and continues to increase in G2 and M phases, thus being highly expressed in rapidly dividing cells. Some anticancer drugs such as anthracyclines, etoposide and mitoxantrone act by targeting *Topo-IIa* and preventing it from religating nicks in DNA, thus introducing strand breaks.[14,15] *Topo-IIa* expression has been found to correlate with clinical outcome in various malignancies, but the findings of various studies are contradictory, where high *Topo-IIa* expression is correlated with a favorable outcome in some studies,[16–19] while having an adverse outcome in others.[20–23] For example, in acute lymphoblastic leukemia (children n=65), an increased *Topo-IIa* expression correlated with daunorubicin resistance as part of the FRALLE-93 protocol.[20] Thus, it is imperative that the chemotherapy outcome should be studied in relation to molecular markers given above to draw a holistic picture. Hence, this study was designed to comprehensively analyze the role of *hENT1*, *dCK*, *CDA*, *dCMPD* and *Topo-IIa* gene expression in response to standard 3+7 AML chemotherapy regimen.

Materials and methods

Patient induction, sample collection and real-time/quantitative PCR

A total of 90 patients diagnosed with AML were recruited at National Institute of Blood Disease & Bone Marrow Transplantation (NIBD&BMT) Karachi, during September 2011 to March 2017. All patients, who received cytarabine 200 mg/m^2/day for 7 days and daunorubicin 45 mg/m^2/day for 3 days, were included. Complete remission (CR) was defined as blast cells <5% in bone marrow and no blast cells in peripheral blood. Patients remained under observation and were assessed for CR documentation during the days 21–28 after the first cycle of induction chemotherapy.

The study was approved by the Ethical Review Board at NIBD&BMT in accordance with the Declaration of Helsinki. All patients provided written informed consent to participate in this research. Any participant under the age of 18 had parental or legal guardian written informed consent confirmed.

Patient bone marrow and peripheral blood samples were collected separately and were used to study the expression of *hENT1*, *dCK*, *CDA*, *dCMPD* and Topo II. Detailed information about sample collection and storage, RNA extraction, reverse transcription reaction and quantitative real-time PCR (qPCR) were previously reported.[24] Briefly, we enriched the leukemia blasts with the Ficoll gradient method.[24] The peripheral blood mononuclear cells layer yielded cells overwhelmingly populated by blast cells. Other mononuclear cells could be seen only sporadically, as confirmed by morphology and flow cytometry. Primers and probes used are: for **hENT1** forward 5′-TGTTTCCAGCCGTGACT-3′, reverse 5′-CAGGCCACATGAATACAG-3′ and probe 5′-/56-FAM/CA GCA CCT G/ZEN/G GAA CGTTAC TT/3IABkFQ/-3′; for **dCK** forward 5′-TGCAGGGAAGTCAACATT-3′, reverse 5′-TCCCACCATTTTTCTGAG-3′ and probe 5′-/56-FAM/TA AAC AAT T/ZEN/G TGT GAA GATTGG GAA G/3IABkFQ/-3′; for **CDA** forward 5′-GGAGGCCAAGAAGTCAG-3′, reverse 5′-GACGGCCTTCTGGATAG-3′ and probe 5′-/56-FAM/CA ACA TAG A/ZEN/A AAT GCC TGCTAC CC/3IABkFQ/-3′; for **dCMPD** forward 5′-AATGGGTGCAGTGATGAC-3′, reverse 5′-CTTAGCGCATTCATTACAAG-3′ and probe 5′-/56-FAM/AT CAT GAA C/ZEN/A AAA ATT CGACCG AT/3IABkFQ/-3′; for **Topo-IIα** forward 5′-AGTCGCTTTCAGGGTTCTTGAG-3′, reverse 5′-TTTCATTTACAGGCTGCAATGG-3′ and probe 5′-/56-FAM/CC CTT CAC G/ZEN/A CCG TCA CCATGG A/3IABkFQ/-3′, and for **GAPDH** forward 5′-GAAGGTGAAGGTCGGAGTCA-3′, reverse 5′-GAAGATGGTGATGGGATTTC-3′, and probe 5′-(FAM)/56-JOEN/CC GAC TCT T/ZEN/G CCC TTCGAA C/3IABkFQ/ (TAMRA)-3′.[25,26]

Statistical analyses

Statistical analyses were done using SPSS version 19.0 (IBM Corporation, Armonk, NY, USA). Data are presented as frequencies and percentages, or median and interquartile ranges where applicable. Chi-squared tests were used to analyze the differences between the groups. Spearman's correlations (r_s) were computed to determine the relationships between different gene expressions. The Kaplan–Meier survival analysis (log-rank test) was carried out to explore the survival according to gene expression. Only a *P*<0.05 was considered significant.

Results

Baseline characteristics

Ninety AML patients were analyzed in this study. Baseline data, such as age, gender and ethnic distribution, AML subtype, myeloperoxidase status, mutations, translocations and karyotyping are given in Table 1, which shows that CR was achieved by 56 patients while 34 were resistant to chemotherapy. A relapse was reported in 19 patients who had achieved CR. Thus, 37 patients who achieved remission and did not relapse during the study period (persistent remission) were labeled as good responders to therapy (41%), whereas all those patients who showed either little or no remission after chemotherapy or relapsed later were grouped together as poor responders (n=53; 59%). One-year disease-free survival (DFS) and overall survival (OS) status was calculated. Since gene expression data were not normally distributed, median and IQRs are provided according to various groups, as shown in Table S1.

Chi-squared and Fisher exact tests

Grouping variables were dichotomized for meaningful analysis, ie, gender (male and female), AML classification (APML being good prognostic, all others being poor prognostic), myeloperoxidase status (negative, positive), remission status (relapse, persistent remission), final therapeutic response (poor, good) and survival status (dead, alive). Nonparametric variables were compared by chi-squared or Fisher's exact test (Table 2). AML other than APML had a higher relapse rate (*P*=0.012), poor final therapeutic response (*P*<0.001) and a trend of higher mortality (*P*=0.05). In addition, patients with relapse and poor therapeutic outcome (resistant + relapsed) had higher mortality (*P*<0.001 for both groups). FLT3-positive patients had a poor therapeutic outcome when compared with FLT3-negative patients (*P*=0.03).

Since the gene expression data were not normally distributed, we stratified it as either low or high expression. If the relative gene expression was up to one-fold, the patients were labeled low expressers, and if more than one, high expressers. Chi-squared or Fisher's exact test was done to compare low or high expression between relapse vs remission, poor vs good therapeutic response and dead vs alive (survival) groups (Table 3). Among good responders, a trend of high *hENT1* expression (bone marrow, *P*=0.07; peripheral blood, *P*=0.05) but lower marrow *dCMPD* expression (*P*=0.043) was observed when compared with poor responders. Although bone marrow *Topo-IIa* showed a trend of being higher among

Table 1 Baseline characteristics of the study population (n=90)

Parameters (n=90)	**N**	**Percent**
Age groups, years		
<15	3	3.3
15–40	62	68.9
41–60	24	26.7
>60	1	1.1
Gender		
Male	66	73.3
Female	24	26.7
AML classification (WHO)		
APML (M3)	17	18.9
AML without maturation (M1)	15	16.7
AML with maturation (M2)	44	48.9
Others	8	8.9
Translocation 6:9	2	
AML with minimal differentiation (M0)	2	
Acute myelomonocytic leukemia (M4)	2	
Acute panmyelosis with fibrosis	1	
Myeloid proliferations related to Down syndrome	1	
Unknown	6	6.7
MPO status		
Negative	14	15.6
Positive	62	68.9
Unknown	14	15.6
FLT3 mutation		
Negative	35	38.9
Positive	7	7.8
Unknown	48	53.3
NPM1 mutation		
Negative	13	14.4
Unknown	77	85.6
Karyotyping		
Unfavorable	18	20.0
Favorable t(15:17)	7	7.8
Normal	24	26.7
Unknown	41	45.6
Therapeutic response		
Resistant	34	37.8
Relapse	19	21.1
Persistant remission	37	41.1
Final outcome		
Poor (resistant + relapse)	53	58.9
Good (persistent remission)	37	41.1
Survival status		
Died	42	46.7
Alive	44	48.9
Unknown	4	4.4

Abbreviations: AML, acute myeloid leukemia; MPO, myeloperoxidase.

Table 2 Comparison between groups according to baseline characteristics

Parameters	Groups	N	χ^2 value	*P*-value	OR	95% CI	
						Lower	Upper
AML classification (APML vs others)							
Gender	Male	61	0.16	0.69	1.29	0.37	4.45
	Female	23					
MPO	Negative	12	13.69	<0.001	11.20	2.61	47.99
	Positive	61					
FLT3	Negative	32	0.01	1.00	1.11	0.11	11.33
	Positive	7					
MLL	Negative	9			Invalid		
	Positive	5					
Remission status	Relapse	16	6.32	0.01	0.10	0.01	0.81
	Remission	34					
Survival status	Dead	39	4.28	0.05	0.29	0.08	0.98
	Alive	42					
Final response	Poor	50	15.51	<0.001	0.09	0.02	0.35
	Good	34					
MPO status (negative vs positive)							
Gender	Male	54	0.38	0.54	0.68	0.20	2.32
	Female	22					
Classification	APML (M3)	11	13.69	<0.001	11.20	2.61	47.99
	Others	62					
FLT3	Negative	30	1.44	0.56	Invalid		
	Positive	6					
MLL	Negative	9	0.60	1.00	Invalid		
	Positive	5					
Remission status	Relapse	18	5.18	0.03	0.11	0.01	0.97
	Remission	29					
Survival status	Dead	38	1.05	0.31	0.53	0.16	1.81
	Alive	36					
Final response	Poor	47	8.05	0.01	0.18	0.05	0.64
	Good	29					
Remission (relapse vs persistent remission)							
Gender	Male	45	0.04	1.00	0.88	0.22	3.46
	Female	11					
Classification	APML (M3)	15	6.32	0.02	0.10	0.01	0.81
	Others	35					
MPO status	Negative	11	5.18	0.03	0.11	0.01	0.97
	Positive	36					
FLT3	Negative	26			Invalid		
	Positive	–					
MLL	Negative	7	0.74	1.00	Invalid		
	Positive	2					
Survival status	Dead	19	23.92	<0.001	28.13	6.16	128.36
	Alive	34					
Final response	Poor	19	56.00	<0.001	Invalid[a]		
	Good	37					
Final response (poor vs good)							
Gender	Male	66	1.93	0.16	0.49	0.18	1.35
	Female	24					
Classification	APML (M3)	17	15.51	<0.001	0.09	0.02	0.35
	Others	67					
MPO status	Negative	14	8.05	0.01	0.18	0.05	0.64
	Positive	62					
FLT3	Negative	35	5.17	0.03	Invalid		
	Positive	7					

(*Continued*)

Table 2 (*Continued*)

Parameters	**Groups**	**N**	**χ^2 value**	***P*-value**	**OR**	**95% CI**	
						Lower	**Upper**
MLL	Negative	10	0.13	1.00	0.67	0.08	5.88
	Positive	5					
Remission status	Relapse	19	56.00	<0.001	Invalid[a]		
	Remission	37					
Survival status	Dead	42	30.93	<0.001	20.36	6.07	68.26
	Alive	44					
Survival status (dead vs alive)							
Gender	Male	63	1.82	0.18	0.51	0.19	1.36
	Female	23					
Classification	APML (M3)	16	4.28	0.05	0.29	0.08	0.98
	Others	65					
MPO status	Negative	13	1.05	0.31	0.53	0.16	1.81
	Positive	61					
FLT3	Negative	33	1.56	0.41	0.33	0.06	1.97
	Positive	7					
MLL	Negative	10	0.50	0.58	0.43	0.04	4.64
	Positive	4					
Remission status	Relapse	19	23.92	<0.001	28.13	6.16	128.36
	Remission	34					
Final Response	Poor	52	30.93	<0.001	20.36	6.07	68.26
	Good	34					

Note: [a]Because all relapse in poor and all remission in good category. All chi-squared values *df* =1

Table 3 Comparison between groups according to gene expression

Parameters	**Groups**	**N**	**χ^2 value**	***P*-value**	**OR**	**95% CI**	
						Lower	**Upper**
Remission status (relapse vs persistent remission)							
Bone marrow:							
dCK	Low (<1)	42	0.26	0.68	0.67	0.14	3.24
	High (>1)	8					
CDA	Low (<1)	27	0.00	0.95	0.96	0.29	3.24
	High (>1)	23					
dCMPD	Low (<1)	40	2.38	0.12	0.33	0.08	1.39
	High (>1)	10					
hENT1	Low (<1)	23	1.38	0.24	0.47	0.13	1.67
	High (>1)	27					
dCK/CDA	Low (<1)	37	0.40	0.73	1.60	0.37	6.91
	High (>1)	13					
Topo-IIα	Low (<1)	4	0.83	0.57	2.54	0.32	19.96
	High (>1)	46					
Peripheral blood:							
dCK	Low (<1)	35	1.37	0.30	2.66	0.50	14.25
	High (>1)	11					
CDA	Low (<1)	24	0.01	0.91	1.07	0.31	3.68
	High (>1)	22					
dCMPD	Low (<1)	32	1.15	0.33	2.20	0.51	9.51
	High (>1)	14					
hENT1	Low (<1)	19	3.21	0.07	3.15	0.88	11.31
	High (>1)	27					
dCK/CDA	Low (<1)	33	1.51	0.22	0.44	0.12	1.66
	High (>1)	13					

(*Continued*)

Table 3 (*Continued*)

Parameters	Groups	N	χ^2 value	*P*-value	OR	95% CI	
						Lower	**Upper**
Topo-IIα	Low (<1) High (>1)	8 38	13.28	**0.001**	26.25	2.81	245.52
Final response (poor vs good)							
Bone marrow:							
dCK	Low (<1) High (>1)	66 16	1.06	0.30	0.55	0.17	1.74
CDA	Low (<1) High (>1)	43 39	0.08	0.77	0.88	0.37	2.11
dCMPD	Low (<1) High (>1)	61 21	4.11	**0.04**	0.32	0.11	0.99
hENT1	Low (<1) High (>1)	33 49	3.18	0.07	0.44	0.18	1.09
dCK/CDA	Low (<1) High (>1)	63 19	1.00	0.32	1.69	0.60	4.74
Topo-IIα	Low (< 1) High (>1)	5 77	0.02	1.00	1.13	0.18	7.12
Peripheral blood:							
dCK	Low (<1) High (>1)	61 16	2.15	0.14	2.28	0.75	6.97
CDA	Low (<1) High (>1)	43 34	0.38	0.54	1.33	0.53	3.33
dCMPD	Low (<1) High (>1)	49 28	0.02	0.90	0.94	0.36	2.42
hENT1	Low (<1) High (>1)	35 42	3.65	0.06	2.50	0.97	6.47
dCK/CDA	Low (<1) High (>1)	57 20	0.31	0.58	0.74	0.26	2.13
Topo-IIα	Low (<1) High (>1)	14 63	7.80	**0.01**	11.82	1.46	95.85
Survival status (dead vs alive)							
Bone marrow:							
dCK	Low (<1) High (>1)	64 15	0.31	0.58	0.73	0.23	2.24
CDA	Low (<1) High (>1)	42 37	0.02	0.88	1.07	0.44	2.59
dCMPD	Low (<1) High (>1)	58 21	0.35	0.55	0.74	0.27	2.01
hENT1	Low (<1) High (>1)	31 48	0.05	0.82	1.11	0.45	2.74
dCK/CDA	Low (<1) High (>1)	60 19	0.23	0.64	1.29	0.45	3.65
Topo-IIα	Low (<1) High (>1)	5 74	0.10	1.00	0.74	0.12	4.71
Peripheral blood:							
dCK	Low (<1) High (>1)	59 15	1.47	0.23	2.07	0.63	6.79
CDA	Low (<1) High (>1)	41 33	0.08	0.78	1.14	0.46	2.86
dCMPD	Low (<1) High (>1)	47 27	0.14	0.71	1.20	0.46	3.10
hENT1	Low (<1) High (>1)	33 41	2.52	0.11	2.12	0.83	5.39

(*Continued*)

Table 3 (*Continued*)

Parameters	Groups	N	χ^2 value	*P*-value	OR	95% CI	
						Lower	Upper
dCK/CDA	Low (<1)	55	0.29	0.59	0.75	0.26	2.13
	High (>1)	19					
Topo-IIα	Low (<1)	14	0.14	0.71	0.80	0.25	2.59
	High (>1)	60					

Notes: All chi-squared values *df* =1.

Abbreviations: CDA, cytidine deaminase; dCK, deoxycytidine kinase; dCMPD, deoxycytidine monophosphate deaminase; hENT1, human equilibrative nucleoside transporter 1; Topo-IIα, topoisomerase IIα.

good responders, it did not reach significance. However, such significance for *Topo-IIa* expression was achieved in peripheral blood samples of good responders (*P*=0.006). Subgroup analysis showed that peripheral blood *Topo-IIa* expression was higher in persistent remission vs relapse group (*P*=0.001).

No significant difference was found in the gene expression of *dCK* and *CDA* among these clinical outcome groups.

OR was computed, which showed that patients with low *Topo-IIa* expression in peripheral blood relapsed more often (OR: 26.25) and had poorer therapeutic response (OR: 11.8) than those with higher expression. Patients with lower *dCMPD* expression in bone marrow were likely to have good response (OR: 3.1).

Nonparametric correlation – Spearman's Rho (r_s)

Correlation was computed among the expression of genes in bone marrow and peripheral blood (Table 4). A positive correlation was observed between the expression in bone marrow and peripheral blood for each gene. There was a positive correlation between *dCK* and *dCMPD* as well as *hENT1* in respective bone marrow and peripheral blood samples. Both bone marrow and peripheral blood *dCK* expression positively correlated with *dCK/CDA* ratio in both bone marrow and peripheral blood. *CDA* expression positively correlated with *hENT1* and *Topo-IIa* expression in respective sample types. *dCMPD* expression positively correlated with *hENT1* and *Topo-IIa* expression in respective sample types. In addition, *Topo-IIa* correlated positively with *dCK/CDA* ratio.

OS

OS was calculated for 6 and 12 months by Kaplan–Meier analysis and is presented in Figure 1. Better OS at 12 months was noted for APML (*P*=0.031), whereas poor OS at 6 and 12 months was seen in those with FLT3 mutation when compared with those without it (*P*=0.003 and *P*=0.001, respectively). A trend of higher *Topo-IIa* expression in peripheral blood with better DFS was observed (*P*=0.09). No significant differences were observed for higher and lower expression of other genes regarding 6- or 12-month OS.

DFS

Figure 2 shows better 12-month DFS among high *CDA* expressors (*P*=0.032) in bone marrow. Better DFS was found with high bone marrow *Topo-IIa* expression at both 6 (0.04) and 12 months (*P*=0.04). A trend of high *Topo-IIa* expression in peripheral blood with better DFS was also observed (*P*=0.08).

Discussion

We observed that high *Topo-IIa* expression is strongly associated with a better CR rate in AML patients treated with standard dose 3+7 induction chemotherapy comprising Ara-C and daunorubicin, and thus translates into low relapse and better DFS, whereas high dCPMD expression was associated with poor chemotherapy response. In agreement with other studies, we observed that patients with APML and absent FLT3 mutation had better therapeutic response and survival.

Although WHO and European Leukemia Net classifications have included many factors determining the clinical outcome in terms of response and survival, the picture is far from complete. Remission rates remain poor and many patients do not survive, pointing to the possibility of incompletely treated disease with chemotherapy. Multiple mechanisms can potentially be attributed to a partial or absent response to chemotherapy. These may include alterations in influx and efflux of the chemotherapeutic drugs, activation and inactivation of the drugs, drug target availability and ultimately the efficiency of apoptotic machinery. These factors are not yet part of any AML therapeutic guideline partly due to conflicting data. The current study is an attempt to clarify the role of these mechanisms in association with therapeutic outcome in AML patients treated with Ara-C and daunorubicin.

Table 4 Spearman correlation matrix

Variables		dCK_M	dCK_B	CDA_M	CDA_B	dCMPD_M	dCMPD_B	hENT1_M	hENT1_B	Topo-IIα_M	Topo-IIα_B	dCK/CDA_M	dCK/CDA_B
dCK_M	rs	1.00	0.52^{a}	0.15	0.04	0.37^{a}	0.13	0.41^{a}	0.30^{b}	0.13	0.04	0.65^{a}	0.30^{b}
	n	82	69	82	69	82	69	82	69	82	69	82	69
dCK_B	rs		1.00	0.00	0.09	0.09	0.35^{a}	0.15	0.47^{a}	–0.11	0.15	0.43^{a}	0.73^{a}
	n		77	69	77	69	77	69	77	69	77	69	77
CDA_M	rs			1.00	0.54^{a}	0.29	0.01	0.48^{a}	0.00	0.41^{a}	–0.24	–0.55^{a}	–0.29
	n			82	69	82	69	82	69	82	69	82	69
CDA_B	rs				1.00	0.13	0.36^{a}	0.18	0.41^{a}	0.09	0.23^{b}	–0.28^{b}	–0.48^{a}
	n				77	69	77	69	77	69	77	69	77
dCMPD_M	rs					1.00	0.38^{a}	0.59^{a}	0.19	0.23^{b}	0.10	0.03	–0.09
	n					82	69	82	69	82	69	82	69
dCMPD_B	rs						1.00	0.17	0.68^{a}	0.00	0.30^{b}	0.14	0.02
	n						77	69	77	69	77	69	77
hENT1_M	rs							1.00	0.29^{b}	0.30^{b}	0.00	–0.08	–0.05
	n							82	69	82	69	82	69
hENT1_B	rs								1.00	–0.06	0.46^{a}	0.37^{a}	0.09
	n								77	69	77	69	77
Topo-IIα_M	rs									1.00	0.28^{b}	–0.18	–0.14
	n									82	69	82	69
Topo-IIα_B	rs										1.00	0.27^{b}	–0.06
	n										77	69	77
dCK/CDA_M	rs											1.00	0.45^{a}
	n											82	69
dCK/CDA_B	rs												1.00
	n												77

Notes: ^{a}*P*-value <0.01. ^{b}*P*-value <0.05 but more than 0.01.

Abbreviations: B, peripheral blood; CDA, cytidine deaminase; dCK, deoxycytidine kinase; dCMPD, deoxycytidine monophosphate deaminase; hENT1, human equilibrative nucleoside transporter 1; Topo-IIα, topoisomerase IIα. M, bone marrow; rs, Spearman correlation coefficient; n, sample size.

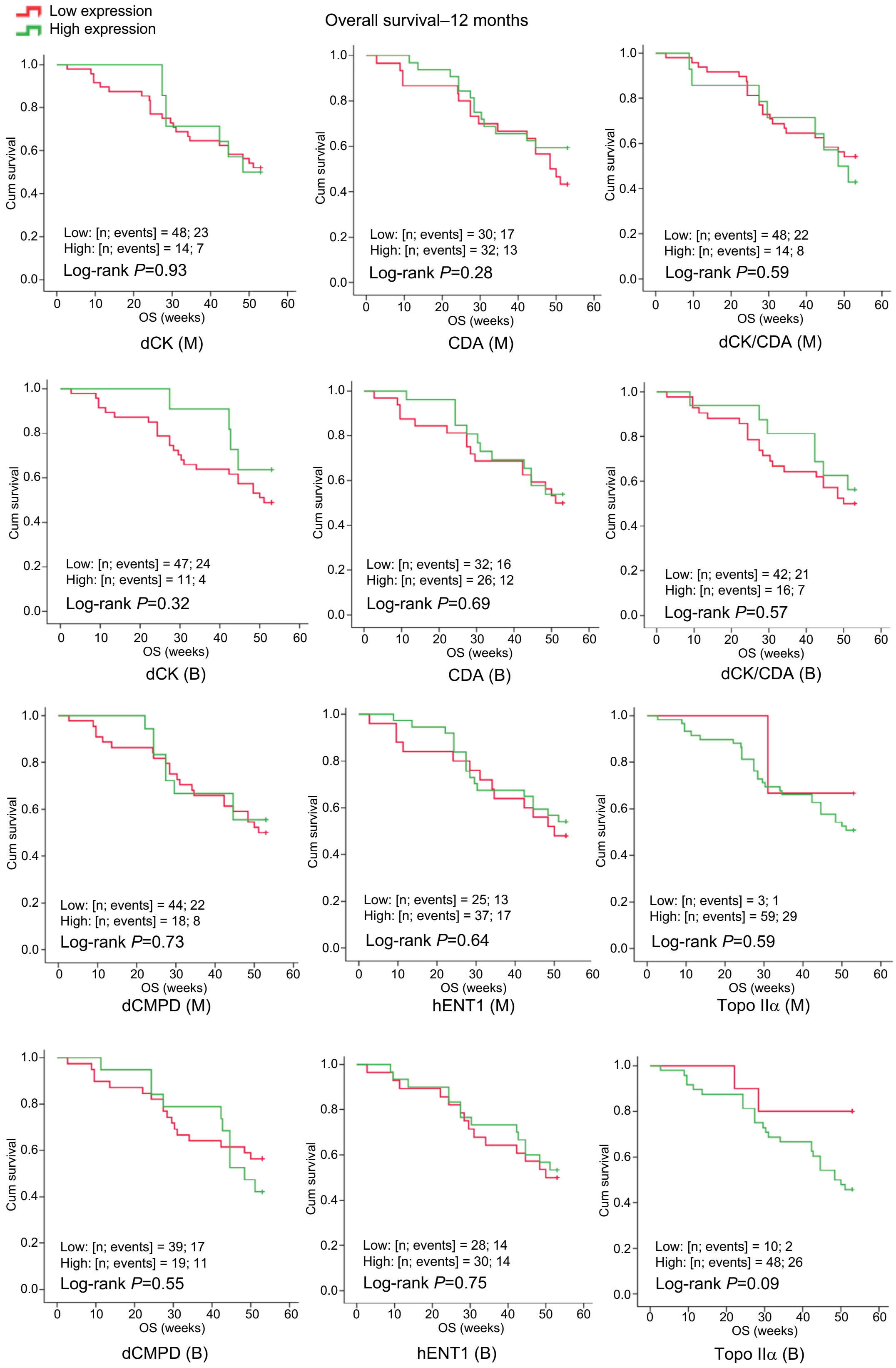

Figure 1 Kaplan–Meier curves showing OS in relationship to dCK, CDA, dCK/CDA, dCMPD, hENT1 and Topo-IIα expression in bone marrow (M) and peripheral blood (B).
Abbreviations: CDA, cytidine deaminase; Cum, cumulative; dCK, deoxycytidine kinase; dCMPD, deoxycytidine monophosphate deaminase; hENT1, human equilibrative nucleoside transporter 1; OS, overall survival; Topo-IIα, topoisomerase IIα.

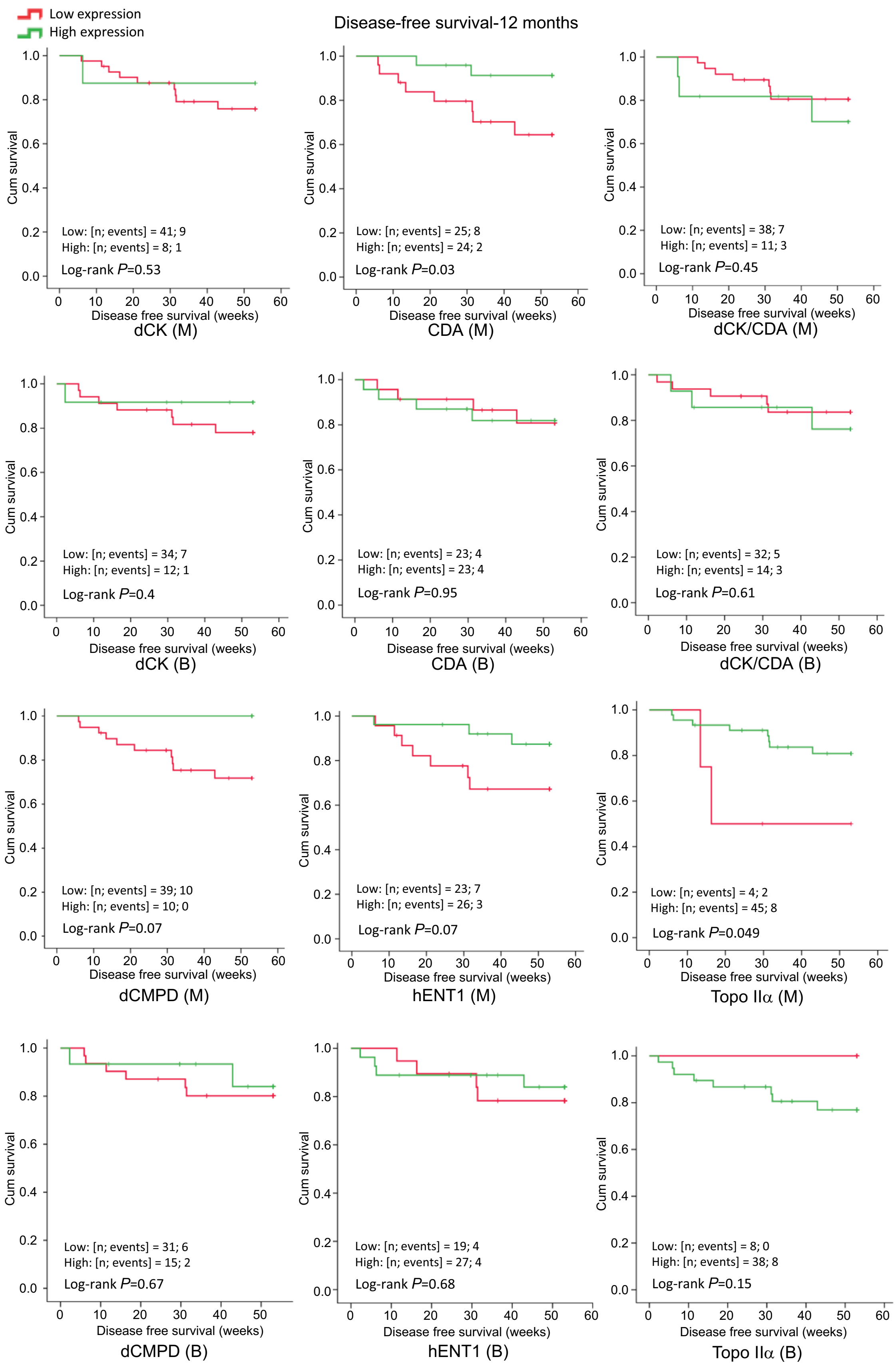

Figure 2 Kaplan–Meier curves showing DFS in relationship to dCK, CDA, dCK/CDA, dCMPD, hENT1 and Topo-IIα expression in bone marrow (M) and peripheral blood (B).

Abbreviations: CDA, cytidine deaminase; Cum, cumulative; dCK, deoxycytidine kinase; dCMPD, deoxycytidine monophosphate deaminase; DFS, disease free survival; hENT1, human equilibrative nucleoside transporter 1; Topo-IIα, topoisomerase IIα.

Expression of Ara-C transporter *hENT1* and metabolizing enzymes *dCK*, *CDA* and *dCMPD*

Ara-C is transported inside the cell by *hENT1*[5] and increased retention of Ara-C in leukemic cells has been associated with longer duration of CR in patients with AML.[27] Low and high expression of *hENT1* have been reported as a predictor of Ara-C resistance in AML[10,28] and sensitivity in acute lymphocytic leukemia,[9] respectively. Some solid organ tumors have shown interesting results. For example, some degree of *hENT1* protein expression has been found associated with increased OS and DFS in pancreatic cancer treated with gemcitabine (a pyrimidine nucleoside analog) when compared with no *hENT1* expression.[29–31] It has also been shown in vitro that *hENT1* expression positively correlated with IC_{50} of gemcitabine, suggesting a direct role of *hENT1* expression in cancer cell sensitivity in a non-small-cell lung cancer cell line exposed to gemcitabine.[32] Greenhalf et al[31] inferred that pancreatic cancer patients with no *hENT1* expression should not be treated with gemcitabine. Similarly, higher expression of *hENT1* has been found associated with prolonged survival or better therapeutic response in patients with advanced biliary tract cancer who were treated with gemcitabine.[33]

In a recent study of hematological malignancy, high-risk Myelodysplastic syndrome patients treated with decitabine (another pyrimidine analog) also suggested a similar outcome.[34] We observed that in AML patients treated with the standard 3+7 regimen, a trend of higher *hENT1* expression was seen among good responders, although it did not reach statistical significance (bone marrow, P=0.07; peripheral blood, P=0.05). This inability to reach statistical significance could be partly explained based on sample size, because another study with a higher sample size reported a shorter DFS among *hENT1*-deficient AML patients (n=123) treated with Ara-C dosage similar to our study.[28]

Once Ara-C is inside the cells, *dCK*, a cytoplasmic enzyme, activates Ara-C by phosphorylating it, which is then incorporated in the newly synthesized DNA, while *CDA* inactivates it. Abraham et al[35] have described Ara-C resistance index, which is denoted as ΔCt (DCK × ENT1)/ΔCt *CDA*. They found that resistance index values were significantly higher in resistant patients compared with sensitive patients. In contrast, we have observed that *dCK* and *CDA* expression was not associated with response to chemotherapy; however, we had a smaller sample size but more rigorous method of gene expression calculation (ΔΔCt), which might explain the difference. Regarding the inactivators of Ara-C, we have shown that expression of bone marrow *dCMPD* was significantly higher in poor therapeutic response, with OR=3 (Table 3). Paradoxically, a higher *CDA* expression in bone marrow (instead of anticipated lower expression) was found significantly associated with better DFS. At present, the significance of this isolated finding is unclear. This could be a chance finding because no other subgroup analysis could support this observation. Hence, it needs to be explored further.

In contrast, Achiwa et al[32] did not find any correlation of *dCK* expression with IC_{50} of gemcitabine in a non-small-cell lung cancer line. Similarly, many studies have shown no significant association of the gene expression of *dCK*, *CDA* and *hENT1* with clinical outcome or survival in AML (n=123 and 42)[28,36] as well as a variety of other cancers such as myelodysplastic syndrome (n=98)[34] and biliary tract cancer (n=28).[33] Our study shows a similar trend of *dCK* or *CDA* expression with regard to patient survival or chemotherapy outcome.

We observed that bone marrow and peripheral blood *hENT1* expression correlated with each other and with *dCK*, *CDA*, *dCMPD* and *Topo-IIa* positively in respective bone marrow and peripheral blood samples (Table 4). These observations might indicate a synchronized regulation of expression of these genes suggesting differential transcriptomic regulation.

Role of anthracycline target *Topo-IIa*

The expression of Top IIα, being a target molecule for anthracyclines, seems imperative in assessing the response to anthracycline-based chemotherapy. In our study, AML patients were treated with daunorubicin 45 mg/day as part of the standard regimen described above. We observed that higher peripheral blood *Topo-IIa* expression was associated with persistent remission as compared with resistance (P=0.047) or relapse (P<0.001) (Mann–Whitney U-test, data not provided). In addition, patients with higher peripheral blood *Topo-IIa* expression had significantly better DFS at 6 and 12 months. There was no effect of *Topo-IIa* expression on OS. Patients with low *Topo-IIa* expression in peripheral blood had higher odds to develop relapse (OR >26) and poor therapeutic response (OR >11). Thus, availability of *Topo-IIa* is important for a better therapeutic response. *Topo-IIa* expression in peripheral blood was found positively and strongly correlated with *hENT1* expression. This correlation may be explained by a possible upregulation of *Topo-IIa* in response to the drug entering the cell, although the transporter for daunorubicin entry is not well characterized. Some studies have shown, in agreement with our findings that higher *Topo-IIa* expression could predict better outcome in breast cancer patients treated with anthracyclines. In one study, patients were treated with five cycles of 5-flurouracil, epirubicin, cyclophosphamide, which included an anthracycline called epirubicin 75–100 mg/m^2.[18] In another study, it was reported that *Topo-IIa* expression in Her2/neu expressing breast cancer positively influenced response to anthracycline-based chemotherapy.[17] A systematic review of breast cancer patients receiving anthracycline-based chemotherapy has also supported this observation.[16] As described earlier, *Topo-IIa* expression increases during S, G2 and M phases of the cell cycle, and is thus highly expressed in rapidly dividing cells.[14,15] Chemotherapy may kill the cancer cells and lead to senescence of normal tissue.[37] Thus, it can be hypothesized that if *Topo-IIa* is highly expressed it may not only provide a better drug target in malignant cells but also help in subsequent DNA repair in normal tissue damaged by chemotherapy.

However, high expression may be a double-edged sword, as it may dilute the therapeutic effect of anthracyclines by providing abundant topoisomerase function to evade the drug action. Currently, little is known about this intricate balance and much seems to depend on pathology, tissue involved and anthracycline dosing, among other factors. For example, in contrast to our results, in small cell lung cancer patients (n=93) receiving doxorubicin and etoposide, high *Topo-IIa* expression was associated with poor therapeutic outcome and low OS.[21] Similarly, another study conducted on Hodgkin's lymphoma patients receiving anthracycline (doxorubicin or epirubicin; n=238) reported that high expression of *Topo-IIa* was associated with adverse prognosis, whereas low expression had no effect.[38] In patients with colorectal carcinoma (n=228), *Topo-IIa* overexpression was found associated with advanced tumor stage and resistant disease.[23]

However, some studies could not find any significant association of *Topo-IIa* expression with outcome or survival among AML (n=123, semiquantitative RT-PCR)[28] and breast cancer (n=232) treated with anthracyclines.[39]

Similarly, in some studies of AML, *Topo-IIa* expression was not found associated with clinical outcome at all.[28,40] Conflicting results may be due to the use of techniques with different sensitivities. Immunohistochemistry was the technique used in most of these studies to assess *Topo-IIa* expression, but more sensitive techniques are now available.

The prognostic role of MPO has remained controversial.[41] There could be multiple reasons for that. For example, it is important to only consider studies with similar criteria for MPO positivity while exploring its role related to therapeutic outcome. An interesting observation of our study was the association of MPO positivity with poor therapeutic response.

Others have reported that MPO positivity is a favorable prognostic factor, though the effect size is small.[41] A recent study suggests that MPO status may, in fact, be a reflection of DNA methylation status in blast cells, and thus could be an indirect marker that should be interpreted with caution.[42] Thus, the MPO controversy needs further exploration in depth.

The main strength of our study included adoption of a uniform treatment and follow-up protocol as well as inclusion of both bone marrow and peripheral blood. Our study also had limitations. It was a single-center study and may not be representative of the entire population of AML patients. We recommend that this study should be replicated with a larger sample size and inclusion of other hematological malignancies to validate our results regarding gene expression.

Conclusion

AML patients with APML subtype had better CR rate and better OS. FLT3 mutation is a known bad prognostic marker. All patients positive for this mutation could not achieve CR and all were resistant to chemotherapy. A higher *hENT1* expression tended to be associated with better CR rate in these patients. However, this observation may be confirmed by use of a larger sample size. *CDA* and *dCK* expression did not correlate with CR rate. Higher dCPMD expression was associated with poor chemotherapy response. A high *Topo-IIa* expression is strongly associated with better CR rate in AML patients treated with standard dose 3+7 induction chemotherapy comprising Ara-C and daunorubicin, and thus translates into low relapse and better DFS.

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Author contributions

All authors met authorship criteria and made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Gene expression profile, grouped by various factors

Parameters	**N**	***dCK***			***CDA***			***dCMPD***			***hENT1***			***Topo-IIα***			***dCK/CDA***		
		Median	**25**	**75**	**Median**	**25**	**75**	**Median**	**25**	**75**	**Median**	**25**	**75**	**Median**	**25**	**75**	**Median**	**25**	**75**
Bone marrow																			
AML classification																			
APML (M3) with t(15:17)	17	0.03	0.00	0.90	1.29	0.02	5.52	0.77	0.39	1.49	1.71	0.04	3.02	19.27	6.61	123.5	0.35	0.00	1.33
AML without maturation (M1)	14	0.05	0.00	3.10	0.52	0.01	10.85	0.97	0.29	13.47	3.26	0.00	14.44	18.25	8.80	119.4	0.42	0.00	13.44
AML with maturation (M2)	40	0.03	0.00	0.61	0.50	0.06	2.71	0.59	0.10	0.90	1.50	0.02	3.64	19.53	8.86	39.95	0.11	0.00	0.96
Others	11	0.01	0.00	0.29	0.44	0.00	3.17	0.13	0.06	0.44	0.47	0.18	1.08	14.82	2.54	17.49	0.19	0.00	0.96
APML vs others																			
APML (M3)	17	0.03	0.00	0.90	1.29	0.02	5.52	0.77	0.39	1.49	1.71	0.04	3.02	19.27	6.61	123.5	0.35	0.00	1.33
All others (Poor)	61	0.03	0.00	0.67	0.54	0.06	3.28	0.54	0.13	1.15	1.56	0.10	4.10	17.24	8.68	43.57	0.12	0.00	0.98
Myeloperoxidase status																			
Negative	13	0.03	0.00	0.68	1.27	0.04	5.31	0.38	0.08	0.79	0.47	0.04	2.76	15.72	7.74	483.5	0.15	0.01	0.66
Positive	58	0.04	0.00	1.00	0.52	0.01	2.90	0.65	0.26	1.33	2.14	0.33	4.30	17.33	7.03	40.34	0.26	0.00	2.10
Pre or postchemotherapy sample																			
Prechemo	32	0.01	0.00	0.90	0.36	0.01	1.65	0.72	0.13	1.43	1.64	0.04	3.89	13.36	6.84	87.02	0.96	0.01	3.29
Postchemo	50	0.04	0.00	0.67	1.30	0.05	6.23	0.51	0.13	0.98	1.31	0.04	3.61	18.23	7.34	61.95	0.09	0.00	0.96
Remission status																			
Resistant	32	0.04	0.00	1.07	0.88	0.12	4.85	0.68	0.24	1.44	3.11	0.32	4.64	17.17	6.84	93.13	0.18	0.00	0.96
Relapse	15	0.08	0.00	0.82	0.38	0.01	6.76	0.50	0.07	1.13	1.84	0.21	8.76	15.93	7.57	32.37	0.05	0.00	0.96
Remission	35	0.01	0.00	0.53	0.45	0.01	2.54	0.62	0.09	0.86	0.92	0.01	2.43	19.27	6.64	100.8	0.65	0.00	2.02
Survival																			
Dead	37	0.04	0.00	0.90	0.54	0.02	3.51	0.49	0.08	1.24	1.64	0.12	4.02	15.93	6.93	48.48	0.14	0.00	0.98
Alive	42	0.02	0.00	0.61	0.76	0.01	2.89	0.65	0.29	0.94	1.36	0.04	3.75	19.12	8.08	61.95	0.27	0.00	1.77
Blood																			
AML classification																			
APML (M3) with t(15:17)	14	0.56	0.00	1.33	1.22	0.04	2.20	0.60	0.28	1.31	2.22	0.31	3.63	8.55	2.28	18.72	0.65	0.00	0.95
AML without maturation (M1)	13	0.21	0.00	1.61	1.03	0.09	2.90	1.68	0.54	2.98	2.37	0.05	5.66	7.57	2.89	16.03	0.05	0.00	5.21
AML with maturation (M2)	38	0.00	0.00	0.43	0.24	0.01	2.25	0.44	0.02	1.04	0.74	0.01	2.14	6.18	0.37	24.57	0.21	0.00	1.01
Others	12	0.11	0.01	1.04	0.35	0.04	2.35	0.58	0.19	2.21	1.22	0.66	4.11	6.55	3.72	416.2	0.61	0.02	241.9
APML vs others																			
APML (M3)	14	0.56	0.00	1.33	1.22	0.04	2.20	0.60	0.28	1.31	2.22	0.31	3.63	8.55	2.28	18.72	0.65	0.00	0.95
All others (Poor)	58	0.00	0.00	0.66	0.52	0.03	2.45	0.53	0.15	1.52	0.98	0.02	3.23	5.56	1.68	14.55	0.06	0.00	1.46
Myeloperoxidase status																			
Negative	12	0.11	0.00	1.46	1.28	0.05	2.45	0.21	0.00	0.60	0.47	0.01	2.98	6.78	2.91	81.44	0.71	0.02	0.96
Positive	55	0.00	0.00	0.81	0.69	0.03	2.19	0.75	0.29	1.47	1.24	0.07	3.28	7.57	1.99	15.13	0.06	0.00	1.53

(Continued)

Table S1 (*Continued*)

Parameters	**N**	***dCK***			***CDA***			***dCMPD***			***hENT1***			***Topo-IIα***			***dCK/CDA***		
		Median	**25**	**75**	**Median**	**25**	**75**	**Median**	**25**	**75**	**Median**	**25**	**75**	**Median**	**25**	**75**	**Median**	**25**	**75**
Pre or postchemotherapy sample																			
Prechemo	31	0.01	0.00	1.19	0.14	0.01	1.20	0.77	0.23	1.84	1.40	0.03	3.35	7.49	1.83	14.36	0.92	0.00	1.43
Postchemo	46	0.03	0.00	0.66	1.20	0.05	3.94	0.50	0.17	1.20	0.79	0.03	2.95	7.22	1.92	24.57			
Remission status																			
Resistant	31	0.00	0.00	0.34	0.64	0.03	1.56	0.91	0.23	1.84	0.90	0.00	3.21	4.68	1.23	14.36	0.02	0.00	0.96
Relapse	15	0.05	0.00	0.63	0.21	0.01	2.42	0.41	0.17	0.62	0.74	0.02	4.61	1.56	0.24	57.38	0.96	0.00	4.50
Remission	31	0.09	0.00	1.11	0.85	0.03	2.55	0.74	0.17	1.40	1.61	0.41	2.86	9.37	3.90	23.06	0.75	0.02	0.96
Survival																			
Dead	35	0.03	0.00	0.39	0.69	0.05	2.42	0.54	0.22	1.31	0.80	0.01	3.21	5.88	2.04	23.37	0.06	0.00	1.88
Alive	39	0.01	0.00	1.10	0.64	0.01	2.19	0.74	0.24	1.44	1.54	0.07	3.28	7.57	1.83	15.13	0.75	0.00	0.96

Note: The values are given as median and IQR (25th–75th percentile).

Abbreviations: AML, acute myeloid leukemia; *CDA*, cytidine deaminase; *dCK*, deoxycytidine kinase; *dCMPD*, deoxycytidine monophosphate deaminase; *hENT1*, human equilibrative nucleoside transporter 1; *Topo-IIα*, topoisomerase IIα.