The Prognostic Significance of Genetic Polymorphisms of Deoxycytidine Kinase and Cytidine Deaminase on the outcome of Adult Acute Myeloid Leukemia Patients with Cytarabine Based Chemotherapy

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ABSTRACT

Cytarabine (Ara-C) is the mainstay of treatment of acute myeloid leukemia (AML), but still, drug resistance and treatment-related toxicities are the main causes of treatment failure. Single nucleotide polymorphisms (SNPs) of the key genes involved in the metabolic pathway of Ara-C have been reported to affect the clinical outcome, so we aimed to investigate the potential association of SNPs of deoxycytidine kinase [DCK] 201C>T (rs2306744), DCK 360C>G (rs377182313) and cytidine deaminase (CDA) 79A>C (rs2072671) genes in adult Egyptian AML patients with the outcome. Genotyping of the studied SNPs was tested using PCR- RFLP technique in 142 adult de novo AML patients who received standard induction chemotherapy based on cytarabine and doxorubicin (3+7 protocol). The median (range) age of our patients was 38 (20-60) years. The studied SNPs genotypes had no significant influence on treatment response on day 28 of induction therapy. DCK 201 heterozygous CT genotype, DCK 201-T allele, [DCK 201(CC)/DCK 360(CC)/CDA 79(AC)] combination as well as hepatological, nephrological, neurological and hematological toxicities were independent prognostic markers on the survival of our AML patients. Nucleophosmin mutation was associated with the poor prognostic variant DCK 201-T and CDA 79-A alleles. Fms-like tyrosine3 kinase internal tandem duplication (FLT3-ITD) mutation was associated with the wild AA genotype of CDA 79A>C polymorphism, while FLT3-tyrosine kinase domain (TKD) mutation was associated with variant DCK360-G allele. These findings support the idea that the studied SNPs can be used as prognostic markers helping in tailored treatment for AML patients.

Keywords: Acute myeloid leukemia, Cytarabine, Deoxycytidine Kinase, Cytidine deaminase, Prognosis

INTRODUCTION

Acute myeloid leukemia (AML) is a highly heterogeneous disease which represents the most common type of acute leukemia in adults. Despite the improvements in supportive care management, and risk stratification that helped to improve established therapies, still, the overall survival (OS) of those patients remains suboptimal.[1]

Cytarabine (1-β-arabinofuranosylcytosine [Ara-C]) a deoxycytidine nucleoside analog is the cornerstone of treatment of AML.[2] Ara-C is transported into cells via nucleoside transporters comprising solute carrier family 29 member 1 (SLC29A1).[3] This is followed by the phosphorylation of intracellular Ara-C by deoxycytidine kinase (DCK) into Ara-C monophosphate (Ara-CMP) and ultimately to Ara-C triphosphate which competes for the integration into DNA with deoxycytidine triphosphate and eventually blocking DNA synthesis leading to cell death. Cytidine deaminase (CDA) and deoxycytidylate deaminase are inactivating enzymes that mediate the conversion of Ara-CMP into an inactive structure,[4] and 5´ nucleotidase cytosolic II (NT5C2) antagonizes DCK by mediating the dephosphorylation of Ara-CMP.[5]

The DCK gene is present on chromosome 4q13.3-q21.1, with seven exons on the coding region. Previous studies[6,7] performed sequencing of the coding exons and proximal (1.5kb) DCK promotor and detected linkage disequilibrium in two regulatory SNPs (201 C>T and 360C>G) that decreased DCK mRNA expression and activity of the enzyme and hence adverse outcome.

The CDA gene is placed on chromosome 1 (p36.2-p35). It was previously reported[8] that three SNPs were recognized of which the A79C/ Lys27Gln isoform has been related to reduced enzyme activity causing decreased deamination of Ara-C in vitro and consequently enhance in vitro cytotoxicity.[9]

The molecular cornerstone of interpatient changeability in the concentrations of intracellular Ara-C triphosphate and accordingly response are attributed to the genetic differences in the key genes involved in the Ara-C metabolic pathway.[10] Various studies of individual single nucleotide polymorphisms (SNPs) in Ara-C metabolic pathway genes have revealed that the genetic background is crucial for the outcome of AML patients.[11-15] Therefore, suggested genetic variants may offer potential markers used in the prognosis of patients receiving Ara-C based regimen aiming for tailored and individualized therapy[16] aiming to overcome resistance to treatment and treatment-related toxicities which are the leading causes of treatment failure in AML.[17]

Due to the rarity of the studies conducted in our population, we aimed to investigate the potential association of SNPs of the Ara-C metabolic pathway genes [DCK 201C>T (rs2306744), DCK 360C>G (rs377182313) and CDA 79A>C (rs2072671)] in adult Egyptian AML patients with the treatment response and cytarabine-related organ toxicities as well as the OS and disease-free survival (DFS) rates.

PATIENTS AND METHODS

Patients

This retrospective and prospective study included 142 newly diagnosed adult AML patients in the period from January 2016 to March 2018 presenting to the outpatient clinic of the Medical Oncology department, National Cancer Institute (NCI), Cairo University. Inclusion criteria included adult (18-60 years) patients with either sex was eligible, not receiving any previous chemotherapy and started induction in NCI. However, exclusion criteria included relapsing AML, acute promyelocytic leukemia, therapy related AML, AML arising on top of chronic myeloid leukemia, and patients who weren't candidates for cytarabine therapy including old age > 60 years or association with other comorbidities as chronic renal failure or cardiac patients. All patients provided informed consent. The study was performed following the declaration of Helsinki and was approved by the Internal Review Board at the faculty of medicine, Cairo University. Genotyping of the studied genes was done as a conjoint work between the clinical pathology departments in NCI and the Faculty of Medicine, Cairo University.

Therapy

All patients received standard induction chemotherapy at the Medical Oncology department, NCI, Cairo university. Induction protocol (standard 3+7) was given as 100 mg/m^2 Ara-C over 24 hours on day 1-7, and 45 mg/m^2 doxorubicin over 30 minutes on day 1-3. On day 14 and day 28 of induction, the patients were assessed for achieving complete remission (CR). CR was defined according to the international recommendations as a bone marrow blast count of less than 5% on morphologic examination, with absolute neutrophil count >1,000/μl, platelets >80/μl and no evidence of extramedullary disease.[18] Once CR was achieved, patients ≤ 60 years old were treated with cycles of high-dose Ara-C consolidation, followed by allogeneic hematopoietic stem cell transplantation when a donor is available. For elderly, refractory, or relapsed cases, salvage therapy was administered.[19] Induction related toxicities were graded per Common Terminology Criteria for Adverse Events (CTCAE version 5.0).[20]

Methods

All Patients were subjected to full history and clinical examination, routine laboratory tests including complete blood picture (CBC), liver, and kidney

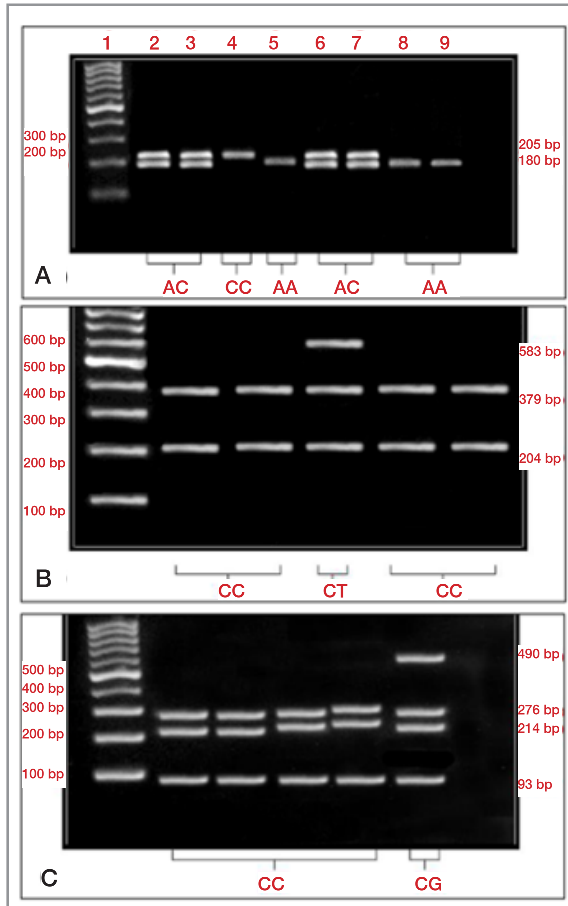

Figure 1. Genotyping of the studied polymorphisms of CDA 79A>C, DCK 201C>T, and DCK 360C>G: (**A**) Digestion of the amplified product of CDA gene by ECO-R1, Lane 1: DNA ladder; Lanes 5, 8 and 9: show wild (AA) genotype; Lanes 2, 3, 6 and 7: show heterozygous genotype (AC); Lanes 4: show variant homozygous genotype (CC); (**B**) Digestion of the amplified product of DCK gene by BglI for the detection of DCK-201 C > T polymorphism, Lane 1: DNA ladder; Lanes 2, 3, 5 and 6: show wild (CC) genotype; Lane 4: show heterozygous genotype (CT); (**C**) Digestion of the amplified product of DCK gene by KasI for the detection of DCK-360C >G polymorphism, Lane1: DNA ladder; Lanes 2, 3, 4 and 5: show wild (CC) genotype; Lane 6: show heterozygous genotype (CG).

function tests were performed. Patients were diagnosed according to the standard techniques including morphological examination of bone marrow smears, immunophenotyping by flow cytometry, conventional cytogenetics, and molecular studies.

Genotyping

Genotyping of polymorphisms of CDA 79A>C (rs 2072671), DCK 201C > T (rs 2306744), and DCK 360C >G (rs 377182313) was carried out using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Genomic DNA was extracted from peripheral blood or bone marrow samples using QIAamp DNA Blood Mini Kit (Qiagen, Germany).

CDA genotyping was performed according to Medina-Sanson et al.,17 using reaction mixture composed of 5 ul Bioline MyTaq ™ Red mix (Bioline, Australia) ,1.25 U of My Taq Red DNA polymerase, 1 ul of 10 pico mole of specific primers (Forward: 5'GACACACCCAAGGGGAGGA3'; Reverse: 3'GACTGTAGGGGCAGTAGGCTGAAT5'), 100 ng genomic DNA and nuclease free water to the final volume of 25 ul. The amplification program used was initial denaturation at 95^{0}C for 3 minutes, followed by 40 cycles of: denaturation at 95^{0}C for 40 seconds, annealing at 63^{0}C for 40 seconds, extension at 72^{0}C for 30 seconds, and final extension at 72^{0}C for 1 minute. The amplified product was detected at 205 bp. Ten microliters of the PCR product were digested with 10 U of restriction enzyme EcoR1 (Thermo scientific, USA) by incubation for 3 hours at 37^{0}C. In case of variant homozygous genotype, restriction site is absent, so the amplified product remains at 205 bp. If the patient is wild type, the enzyme cleaved the DNA at position 79 producing a single band at 180 bp.

DCK genotyping was carried out following szantai et al.[21], amplification of the DCK gene was done using Qiagen Hot start Taq plus DNA polymerase (Qiagen, Germany) in a 25 ul reaction. It included 100 ng genomic DNA, 2.5 ul 1X Coral Load Buffer, 2.5 ul of 10 mmol dNTPs (Qiagen, Germany), 1X Q solution, 1.25 U Hot Start Taq DNA polymerase and 1 ul of 10 pico mole of specific primers Forward: 5'-GCCTTCTCCCCAGATGAGTT-3'; Reverse: 3'GGTGGCCATTCCTTAGTCTTGT5. The thermal cycling conditions were initial denaturation at 95^{0}C for 10 minutes, followed by 35 cycles of: denaturation at 94^{0}C for 60 seconds, annealing at 65^{0}C for 60 seconds, extension at 72^{0}C for 30 seconds, and final extension at 72^{0}C for 10 minutes. The PCR product was detected at 583 bp. The amplified product was digested by 10U of BglI restriction enzyme (New England Biolabs, USA) for 15 minutes at 37^{0}C. In the case of wild type genotype, the enzyme cleaved DNA at posi-

tion 201 producing two fragments at sizes 379 and 204 bp. The restriction site was abolished in case of variant homozygous genotype and the amplicon remained at its original position at 583 bp.

For DCK 360C>G (rs 377182313), 10 ul of the amplified product was digested by 8U of SspDI (Kas I) restriction enzyme (Thermo scientific ,USA). The enzyme cleaved DNA at position 360 producing three bands at 276, 214, and 93 bp in case of wild type genotype. One cleavage site is eliminated giving rise to two fragments at of 490 bp and 93 b if the patient is variant homozygous genotype.

Amplification was done using Biometra T3000 (Biometra, Germany), and running the amplified and digested products was carried out on 2% agarose gel for 30 minutes at 100 volts. Interpretation of the sizes of the bands after digestion of the amplified products is illustrated in Figure 1.

The genotypes of the three polymorphisms of CDA 79A>C, DCK 201C>T, and DCK 360C>G were expressed as wild (AA, CC, CC respectively), heterozygous (AC, CT, CG respectively), and homozygous variant (CC, TT, GG respectively).

Statistical Analysis

Data were analyzed using IBM SPSS advanced statistics (Statistical Package for Social Sciences), version 24 (SPSS Inc., Chicago, IL). Numerical data were described as median and range or mean and standard deviation as appropriate, while qualitative data were described as number and percentage. Chi-square (Fisher's exact) test was used to examine the relation between qualitative variables as appropriate. Survival analysis was done using the Kaplan-Meier method. A Comparison between two survival curves was done using the log- rank test. Multivariate analysis was done by the Cox regression model to test for an independent prognostic effect of statistically significant variables on the univariate level with calculating hazard ratio and its 95% confidence interval. Bonferonni corrections of p-value were done to avoid hyperinflation of type 1 error which arises from multiple testing. The Hardy-Weinberg equilibrium used to determine the allele and genotype frequencies of a population and to prove that the populations are in equilibrium using a Pearson chi square (goodness-of-fit) test. Comparison of genotype and allele frequencies of the studied polymorphisms (DCK 201C<T; DCK 360 C>G and CD 79 A<C) in the studied Egyptian AML patients and other ethnic populations was done using the MedCalc Software Ltd, MedCalc uses the "N-1" Chi-squared test, https://www.medcalc.org/calc/comparison_of_proportions.php accessed at 14/5/2020. A p-value less than or equal to 0.05 was considered statistically significant. All tests were two-tailed. Overall survival (OS) was calculated from the date of diagnosis to date of death or last follow up. Disease-free survival (DFS) was calculated from the date of complete remission to date of relapse, death, or last follow up.

RESULTS

Patients´ Characteristics

One hundred forty-two adult de novo AML patients were enrolled in our study, of whom 75 (52.8%) were males and 67 (47.2%) were females with male to female ratio of 1:1. The median (range) age was 38 (20-60) years. The detailed clinical and laboratory features of our patients are summarized in Table 1.

Genotyping of DCK-201 C>T, DCK-360 C>G and CDA-79 A>C polymorphisms

All our samples were successfully genotyped, the frequencies of the studied genotypes detected in our cohort are illustrated in Table 2, with the highest frequency detected for DCK-201 the (CT) genotype (57%), C-allele (71.5%), for DCK-360 (CC) wild genotype (93.7%), C allele (96.8%) and for CDA-79 AC (51.4%) genotype, and A allele (55.3%). Notably, a high frequency of the following combined polymorphisms [DCK-201 (C<T), DCK- 360 (C<G), CDA-79 (A<C)] was identified: (CT/CC/AC) in 39 (27.5%) patients, then (CC/CC/AC) in 29 (20.4%) patients followed by (CT/CC/AA) in 21 (14.8%) patients. The frequency of the DCK-360 wild genotype (CC) and CDA-79 (A<C), variant (heterozygous, and homozygous) genotypes observed in our studied AML patients were significantly higher compared to the expected frequency with a p-value of < 0.001. However,

Table 1. Patients' characteristics.

Variable	**AML patients (n=142)**
Age (years)*	38 (20-60)
Gender	
Male	75 (52.8)
Female	67 (47.2)
Hb (g/dl)*	7.25 (3.4-13.6)
≤10 g/dl	133 (93.7)
>10 g/dl	9(6.3)
TLC (x10^{3}/μl)*	48.50 (5-358)
≤50x10^{3}/ul [n. (%)]	72 (50.7)
>50x10^{3}/μl [n. (%)]	70 (49.3)
PLT (x10^{3}/μl)*	41 (7-336)
≤40x10^{3}/μl [n. (%)]	71 (50)
>40 x10^{3}/μl [n. (%)]	71 (50)
PB blasts*	40 (10-98)
BM blasts*	70 (20-99)
FAB subtypes	
M0	2(1.4)
M1	29(20.4)
M2	49(34.5)
M4	47(33.1)
M5	15(10.6)
CD34 expression	58(40.8)
t(8;21)	31(21.8)
Inv 16	24(16.9)
FLT3-ITD	
Mutant	19(16.7)
Wild	95(83.3)
FLT3-TKD	
Mutant	5(4.4)
Wild	109(95.6)
NPM mutation (n= 86)	
Mutant	25 (29.1)
Wild	61 (70.9)
Overall survival	
Median (months)	14.6
Disease free survival	
Median (months)	7.0

*Data are presented as number (percentage) unless otherwise indicated; *Median (range).*
Hb: Hemoglobin, TLC: Total leucocytic count, PLT: Platelets, PB: Peripheral blood; BM: bone marrow; FAB: French American British, FLT- ITD,: FMS-like tyrosine kinase 3 internal tandem duplicate, FLT3-TKD: FLT3-tyrosine kinase domain, NPM: Nucleophosmin

DCK-201 genotypes showed a good fit with Hardy Weinberg equilibrium.

There were no statistically significant differences between patients harboring the wild (CC) genotype and those with the heterozygous variant genotype (CT) of DCK-201C<T polymorphism in terms of clinical and laboratory features. Upon comparing the wild (C) and variant (T) alleles of DCK-201C>T polymorphism, patients harboring the variant (T) allele had a significantly higher median hemoglobin concentration (7.2 vs. 8 g/dl, p= 0.041), higher incidence of French American British (FAB) classification for M5 (p< 0.001), and higher association with nucleophosmin (NPM) mutation (39.6% vs. 24.4%; p= 0.042) for the variant (T) and wild (C) alleles respectively.

The mean age at presentation in our patients harboring the wild (CC) genotype was significantly higher than those having the variant heterozygous (CG) genotype of DCK-360 C<T polymorphism (39.3±12.2 vs. 29.7±5.2 respectively; p< 0.001). Nevertheless, no significant differences regarding other prognostic parameters were observed. Similarly, the mean age at presentation in patients carrying the wild allele (C) was significantly higher than those bearing the variant allele (G) (39.9±12.2 vs. 31.7±4.7 respectively, p= 0.001). However, patients with the variant (G) allele showed a significantly higher association with Fms-like tyrosine3 kinase-tyrosine kinase domain (FLT3-TKD) mutation (44.4% vs. 15.5%, p= 0.023) for (G) and (T) alleles respectively, which on multivariate analysis revealed that the risk of FLT3-TKD mutation was 6.941 times higher in patients harboring the variant allele (G) of DCK-360 C<G polymorphism [Odds ratio (OR) 6.941; 95% confidence interval (CI) (1.486-32.419); p= 0.014].

For CDA-79 A<C polymorphism, the incidence of organomegaly was significantly higher in patients harboring the heterozygous (AC) and variant homozygous (CC) genotypes compared to the wild genotype (AA) (52.1, 51.9% and 26.2% respectively; p= 0.018). However, the frequency of FLT3- internal tandem duplication (ITD) mutation was significantly higher in patients carrying the wild (AA) genotype compared to those with the heterozygous (AC) and variant homozygous genotypes (AC and CC) (32.1%, 9.2% ,and 19% respectively; p= 0.023). On multivariate analysis, we detected that the risk of organomegaly was 2.899 times higher in patients harboring the variant heterozygous genotype (AC) [OR 2.899, 95%CI (1.094-7.682);

Table 2. Genotyping and allele frequencies of DCK-201 C>T, DCK-360 C>G and CDA-79 A>C polymorphisms among the studied AML patients

	Polymorphism	AML patients (n= 142)
DCK-201 C>T	Wild (CC)	61 (43)
	VHT (CT)	81 (57)
	C allele	203 (71.5)
	T allele	81 (28.5)
DCK-360 C>G	Wild (CC)	133 (93.7)
	VHT (CG)	9 (6.3)
	C allele	275 (96.8)
	G allele	9 (3.2)
CDA-79 A>C	Wild (AA)	42 (29.6)
	VHT (AC)	73 (51.4)
	VH (CC)	27 (19)
	A allele	157 (55.3)
	C allele	127 (44.7)

DCK: Deoxycytidine kinase, CDA: Cytidine deaminase; VHT: Variant heterozygous; VT: Variant homozygous. Data are presented as number (percentage).

p= 0.032]. Furthermore, the risk of FLT3-ITD mutation was 3.018 times higher in patients harboring the wild genotype (AA) of CDA-79 A<C polymorphism [OR 3.018, 95%CI (1.043-8.727); p= 0.041]. The median (range) hemoglobin level at presentation was 7.4 (3.4-13.6) g/dl in patients carrying the A allele, with a median (range) of 7.2 (3.9-11) g/dl in those with the C allele (p= 0.048). The FAB classification of our patients showed that those harboring the (A) allele had a significantly higher M5 distribution, while patients bearing the variant (C) allele had a significantly higher M2 distribution (p= 0.004). Patients bearing the (A) allele showed significant association with NPM mutation as compared to those with the variant (C) allele (35.1% vs. 21.3% respectively, p= 0.049).

Treatment Outcome

Response to Treatment

All our patients received the 3+7 induction regimen. Eighty from 142 (56.3%) patients achieved CR on day 14, by day 28, 132 (93%) patients achieved CR. Ten (7%) cases did not enter CR and died within the first 6 weeks of induction chemotherapy due to septic shock. There was no statistically significant difference between the different genotypes and alleles of the studied polymorphisms regarding achieving CR or not on day 28 of induction chemotherapy. Also, different combinations of genotypes didn't reveal any significant differences in terms of response to treatment. Notably, we observed that 29/29 (100%) patients carrying [DCK-201(AC)/DCK-360(CC)/CDA-79(CC)] and 15/18 (83.3%) patients harboring [DCK-201(AA)/DCK-360(CC)/CDA-79(CC)] achieved CR on day 28.

Association Between the Studied Genotypes and Cytarabine Toxicity

The median (range) follow up period was 8 (1-39) months. Throughout the follow-up period, all patients had been investigated to analyze the common treatment-related toxicities either neurological, hematological, hepato- or nephrotoxicity by daily clinical examination, complete blood picture, liver, and kidney function tests respectively. Induction related toxicities were graded per Common Terminology Criteria for Adverse Events (CTCAE version 5.0). To facilitate the illustration of different toxicity grades, we classified patients with grade 1 and 2 toxicities as low- grade group and those with grade 3 and 4 toxicities as a high-grade group.

Hematological toxicity was the most common toxicity recognized, as it was detected in the 142 (100%) studied patients, whereas 131(92.3%)

Table 3. Toxicity analysis of different genotypes, and alleles among the studied AML patients

		Febrile neutropenia	Liver impairment Total Bilirubin >1.2mg/dl	Kidney impairment Creatinine >1.3 mg/dl	Neurological toxicity
DCK 201 C<T	CC (n= 81)	28 (46)	17 (27.9)	10 (16.4)	4 (6.6)
	CT (n= 61)	25 (31)	18 (22.2)	17 (21)	1 (1.2)
	P value	0.067	0.440	0.490	0.165
	C (n= 201)	80 (39.4)	42 (21)	30 (15)	8 (3.9)
	T (n= 81)	26 (32.1)	28 (35)	24 (30)	2 (2.5)
	P value	0.250	0.014	0.004	0.543
DCK 360 C<G	CC (n= 133)	48 (36.1)	33 (25)	27 (20.3)	4 (3)
	CG (n= 9)	5 (56.0)	2 (22.2)	0 (0)	1 (11.1)
	P value	0.243	0.861	0.133	0.283
	C (n= 275)	103 (37)	68 (25)	54 (20)	8 (3)
	G (n= 9)	3 (33.3)	2 (22.2)	0 (0)	2 (22.2)
	P value	0.801	0.864	0.140	0.002
CDA 79 A<C	AA (n= 42)	17 (40.5)	11 (26.2)	9 (21.4)	0 (0)
	AC (n= 73)	24 (32.9)	16 (22)	11 15.1)	5 (7)
	CC (n= 27)	12 (44.4)	8 (30)	7 (26)	0 (0)
	P value	0.501	0.702	0.420	*
	A (n= 157)	62 (39.5)	54 (34.4)	40 (25.5)	9 (6)
	C (n= 127)	44 (35.0)	16 (13)	14 (11)	1 (0.8)
	P value	0.401	< 0.001	0.002	0.025

*DCK: Deoxycytidine kinase, CDA: Cytidine deaminase. Data are presented as number (percentage). * No p value due to small number of patients within the strata*

patients had low-grade and 11 (7.75%) had high-grade toxicity. The association between the studied genotypes and common cytarabine toxicities is illustrated in Table 3. Patients carrying the variant allele of DCK 201-T or the wild allele of CDA-79-A had a significantly higher risk of liver impairment (p= 0.014 and < 0.001, respectively) as well as renal impairment (p= 0.004 and 0.002, respectively). Patients with the variant allele of DCK 360-G or the wild allele of CDA 79-A showed a significantly higher association with neurological toxicity (p= 0.002 and 0.025, respectively).

Upon the assessment of the degrees of different forms of drug-related toxicities during the follow-up period, patients having the variant allele of DCK 201-T or the variant allele of DCK 360-G showed a significantly higher association with high- grade liver impairment compared to those carrying the corresponding wild alleles (p= 0.003 and 0.016, respectively). Moreover, those harboring the wild allele of CDA79-A showed a significantly higher association with low-grade liver impairment compared to those having the variant allele (C) (p< 0.001). Patients carrying the variant allele of DCK-201 (T), or the wild allele of CDA-79 (A) showed a significantly higher development of low-grade renal impairment (p= 0.009 and 0.008, respectively). Moreover, no significant differences were observed upon comparing the grades of drug-related toxicities among the different combinations of the studied polymorphisms except for [DCK-201(CT)/DCK-360(CC)/CDA-79(AA)] having an increased risk of high-grade hematological toxicity (p= 0.036).

On multivariate analysis, we noticed an increased risk of renal impairment in patients having the variant allele of DCK 201-T [OR= 2.428, 95% CI (1.313-4.489); p= 0.005], while the risk of neurological toxicity was higher in patients bearing the variant allele of DCK 360-G [OR 14.750, 95% CI

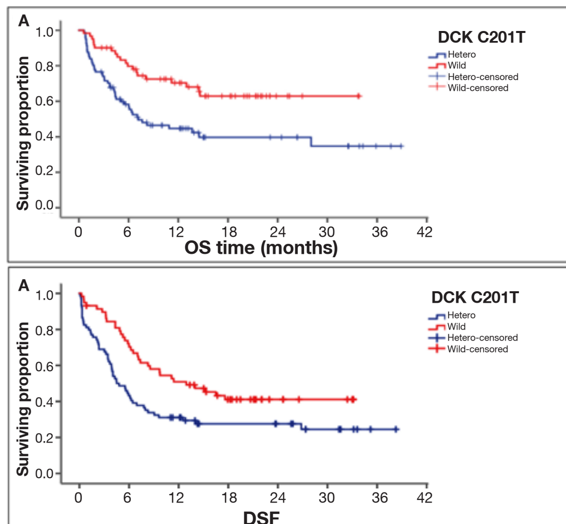

Figure 2. Impact of DCK-201 C>T polymorphism on the survival of our patients: (A) Overall survival of heterozygous variant (CT) versus wild type (CC) genotype (p=0.002), (B) Disease free survival of heterozygous variant versus wild type genotype (p= 0.006).

(2.153-101.047); p= 0.006]. Nevertheless, the risk of liver impairment was higher in patients carrying the wild allele of CDA79-A [OR 3.754, 95%CI (2.008-7.019); p= 0.001). The risk of high-grade hematological toxicity was 3.832 times higher in patients having the [DCK-201(CT)/DCK-360(CC)/CDA-79(AA)] combination [OR 3.832, (1.013-14.488; 95%CI, p= 0.048].

Survival Analysis

The median OS time of our patients was 14.6 months with the cumulative OS (COS) at 12 and 36 months was 55.9% and 44.9% respectively. Patients with liver or kidney impairment had a significantly shorter OS (p< 0.001; p< 0.001, respectively). Otherwise, no significant association between the OS of our patients and other prognostic variables was observed.

The median DFS of our cohort was 7 months with cumulative DFS time at 12 and 36 months of 39.7% and 30.7% respectively. Among the 142 patients, 45 (31.6%) patients had relapsed. Thirty-one of the relapsed patients died one month after the occurrence of relapse. Thirty-four cases died throughout the follow-up period due to drug-related complications with a total number of 65/142 (46%) deaths. Therefore 77/142 (54%) patients were still alive within the 3 years follow up period. No significant association was detected between DFS and different prognostic factors except for liver and renal impairments which showed a significant correlation with an inferior DFS (p= 0.003; p= 0.029, respectively).

Genotyping revealed that patients harboring the heterozygous genotype of DCK-201(CT) had significantly shorter OS and DFS compared to those carrying the wild genotype (CC) (p= 0.002; p= 0.006 respectively), Figure 2. The variant allele (T) of DCK-201 was significantly correlated with decreased OS and DFS compared with the wild allele (C) (p= 0.008; p= 0.014, respectively). However, no significant association between OS, DFS and the polymorphisms of DCK-360 C>G and CDA-79 A>C genotypes was observed.

On studying the impact of single nucleotide polymorphism (SNP) interaction on the outcome of our patients, no statistically significant differences were recognized except for patients carrying the (DCK201(CC)/DCK360(CC)/CDA 79(AC)] combination who had a significantly superior OS and DFS compared to those lacking this combination with p-values of < 0.001 and 0.002, respectively.

Multivariate analysis of OS and DFS using Cox regression hazard model revealed that, liver and kidney impairment were independent adverse parameters on the OS at 3 years [HR (95%CI): 2.041 (1.156-3.605); p= 0.014] and [HR (95%CI): 2.113 (1.328-3.363); p= 0.002] respectively, however, only cases with liver impairment had significantly shorter DFS at 3 years [HR (95%CI): 2.113 (1.328-3.363); p= 0.002]. DCK-201 (CT) genotype was associated with an inferior 3-year DFS and OS compared to those harboring the wild genotype (CC) [HR (95%CI): 1.914 (1.237-2.962); p= 0.004] and [HR (95%CI): 2.230 (1.310-3.797); p= 0.003] respectively. Similarly, patients carrying the variant allele (T) of DCK-201had decreased DFS and OS times at 3 years than those with the wild allele (C) [HR (95%CI): 1.491 (1.081-2.057); p= 0.015] and [HR (95%CI): 2.270 (1.560-3.302); p< 0.001] respectively, Table 4. Furthermore, patients lacking the [DCK-201(CC)/DCK-360(CC)/CDA-79(AC)] combination had significantly shorter 3-year DFS and OS [HR (95%CI): 2.480 (1.372-4.482); p= 0.003) and HR= (95%CI): 4.640 (1.858-11.587); p= 0.001) respectively.

Table 4. Multivariate analysis of overall survival (OS) and disease free survival (DFS) time (Cox regression hazard model)

		Beta coefficient	Standard error	P value	HR (95%C.I.)
DFS	-DCK C201T (heterozygous vs. wild)	0.649	0.223	0.004	1.914 (1.237-2.962)
	-DCK (T vs. C Allele)	0.399	0.164	0.015	1.491 (1.081-2.057)
	Liver impairment (yes vs. no)	0.748	0.237	0.002	2.113 (1.328-3.363)
OS	DCK C201T (heterozygous vs. wild)	0.802	0.272	0.003	2.230 (1.310-3.797)
	-DCK (T vs. C allele)	0.820	0.191	<0.001	2.270 (1.560-3.302)
	Kidney impairment	0.714	0.290	0.014	2.041 (1.156-3.605)
	Liver impairment (yes vs. no)	0.847	0.273	0.002	2.332 (1.366-3.981)

DFS: Disease free survival; OS: Overall survival

DISCUSSION

In the current study, we aimed to investigate the impact of SNPs of the Ara C metabolic pathway genes [DCK 201C>T (rs2306744), DCK 360C>G (rs377182313) and CDA 79A>C (rs2072671)] on the outcome of our patients. We evaluated the allele frequency of the studied SNPs to obtain the first overview of the genetic profile of AML patients in Egypt. Our study showed that DCK 201C>T genotypes distribution was in HWE (p> 0.05), whereas for the DCK 360C>G and CDA 79A>C genotypes distribution were not in agreement with HWE (p< 0.001). Previous studies[11,22] conducted on Caucasians showed that CDA 79A>C genotype distribution was in accordance with HWE, whereas others[15] demonstrated that it was not in agreement with HWE. This variation might be attributed to the ethnic difference of the studied populations.

In our studied Egyptian AML patients, the minor allele frequency (MAF) (DCK 201-T) was statistically significantly higher than in Asians (Thailand)[23] (p= 0.006) and Caucasians (France)[24] and Spain[15] (p< 0.001). However, it was significantly lower than in Mexican Americans[17] (p= 0.034). The MAF (DCK 360-G) was statistically significantly lower compared to Asians (Thailand)[23], Mexican Americans[17], respectively (p< 0.001). The MAF (CDA 79-C) was statistically significantly higher than in Asians (Han Chinese[25], Thailand, Korea, Japan[26], Malay and India[27] (p< 0.001, < 0.001, < 0.001, < 0.001, 0.01 & 0.004) respectively, Americans (Gujarati Indians in Houston, Texas, USA)[28] (p< 0.001), Africans (Kenya and Nigeria)[28] ranging from 0.205 to 0.350. It was also statistically significantly higher than in Caucasians (Caucasians in Seattle area (USA)[29] and Caucasian Americans[26] (0.447 vs. 0.350 & 0.327) (p= 0.037 & 0.018), respectively. However, it was comparable to Mexicans Americans[17] (0.447 vs. 0.333; (0.136) and Caucasians (France[24], Spain[15], Italy[26], and Dutch[30] ranging from 0.350 to 0.490 (0.219, 0.211, 0.155 and 0.065) respectively. The MAFs were different among different ethnic populations suggesting that these polymorphisms could be useful genetic markers. Further detailed studies on different ethnic populations are needed to clarify the geographical distribution of these genetic markers and the possible association between these polymorphisms, ethnicity, and AML treatment outcome.

DCK 201-C and CDA 79-A alleles were significantly associated with lower median hemoglobin levels compared to those carrying the corresponding alleles (p= 0.041; p= 0.049, respectively) which goes in concordance with a meta-analysis that demonstrated a higher incidence of severe anemia with CDA 79A>C variant allele in gemcitabine treated patients.31 However, previous studies[11,15] did not report any significant difference among the baseline characteristics and DCK 201C>T and CDA 79A>C genotypes.

In the current study, we investigated the impact of a single SNP and SNP-SNP interactions on the response to Ara-C based treatment. We demonstrated that the studied SNPs genotypes had no significant

influence on response to treatment on day 28 of induction therapy which goes in agreement with Wan et al[32], who did not detect any statistically significant effect of DCK 201C>T on the treatment outcome. In the same line, previous reports[11,15,22] showed no influence of CDA 79A>C polymorphism on the treatment response in Caucasian AML patients. On the contrary, Shi et al. 7 detected those patients harboring genotypes having DCK 201T/360G haplotype seemed to be correlated with a better clinical response, whereas the DCK those with wild 201C/360C haplotype showed to be associated with inferior drug response in Chinese AML patients. Our study revealed no statistically significant difference between different combinations of genotypes of the studied polymorphisms achieving and not achieving CR on day 28 of induction. If this work was done on a larger group of patients, a significant difference could have been detected.

Identification of patients at risk of severe toxicity at an earlier stage allows for early intervention to reduce morbidity and mortality. CDA 79-A was associated with lower nephrotoxicity grades ($p= 0.008$) and lower hepatotoxicity grades ($p< 0.001$) and this was discordant with Abraham et al.[9] who detected increased cytotoxicity in patients carrying CDA 79A>C polymorphism. Moreover, a previous study 11 reported a higher incidence of Ara C-related higher hepatotoxicity grades in CDA-79 variant genotypes compared to the wild type ($p= 0.03$). In spite of Ara-C being the most important therapeutic agent of AML, patients received a multi-agent therapy including anthracyclines which could have influenced the treatment response and toxicity independent of the examined Ara-C related SNPs, thus more studies are required on larger patient populations to confirm the observed results and to identify other pharmaco-genetic markers that can affect the Ara-C treatment response as well as the influence of other chemotherapeutic agents like anthracyclines on the outcome.

To be noted that NPM mutation was found to be associated with the variant DCK 201-T and CDA 79-A alleles, however, these alleles were found to be of bad prognosis causing hepatological and nephrological toxicities in our studied cohort. FLT3-ITD mutation was found to be associated with the wild AA genotype of CDA 79A>C polymorphism. In agreement with that, the wild AA genotype of CDA 79A>C polymorphism was shown to be of bad prognosis being associated with lower nephrotoxicity grades and lower hepatotoxicity grades. Falk et al.[22] showed that FLT3-ITD mutation was higher in patients harboring CDA-79 AA & AC genotypes compared to those with CC genotype but with no statistically significant difference ($p= 0.660$). FLT3-TKD was found to be associated with the variant DCK360-G allele. In the current study, the DCK 360-G allele was found to be of bad prognosis being associated with higher hepatotoxicity grades. Still more studies on a larger cohort of patients are needed to explore the association of NPM and FLT3 mutations with the studied SNPs which were found to have a bad prognostic impact on AML patients in the current study.

Cox model was applied to identify independent prognostic variables for survival. Liver and renal impairments were adverse prognostic variables on OS ($p< 0.001$, $p< 0.001$) and DFS ($p= 0.003$; $p= 0.029$), respectively. On analyzing the impact of the studied SNPs on the survival of our patients, it revealed that cases harboring DCK 201 heterozygous CT genotype and DCK-T allele had an inferior DFS ($p= 0.006$; $p= 0.014$) and OS ($p= 0.002$; $p= 0.008$), however, no statistically significant effect on DFS and OS times was detected between different DCK 360 genotypes or alleles. On the contrary, Shi et al. 7 reported an association between genotypes [DCK 201 (CC) and DCK 360 (CC)] and poor clinical outcome in AML patients treated with Ara-C-based chemotherapy in a Chinese population. Also, a previous study[17] conducted on a cohort of Mexican pediatric AML patients and showed that the outcome was significantly worse in DCK 201 wild CC genotype [OS ($p= 0.001$) and DFS ($p= 0.001$). However, our results might be attributed to the genetic association of DCK201-T with nephrotoxicity and hepatoxicity proved to be independent predictors of the risk of death in our study. we concluded that this inferior outcome is strongly associated with the drug-related toxicity in patients harboring the DCK 201-T allele.

In the present study, no significant effect of CDA 79 genotypes or alleles on DFS and OS was observed, which goes in line with previous studies.[11,33]

However, Megias-Vericat et al.[15] demonstrated that CDA 79 heterozygous AC genotype showed lower OS, EFS (event-free survival) and relapse-free survival (RFS) rates at 5 years and this was consistent with previous reports[31] in AML cohorts of Asian adults showing that variant (AC & CC) genotypes were 1.840 more likely to have a shorter OS time when compared to AA wild type in Korean AML patients. On the other hand, Falk et al.[22] did not show any significant effect on OS rates for CDA 79 in Caucasians (Swedish population). However, when stratifying patients according to FLT3 status, they showed that the wild CC genotype was associated with shorter OS in FLT3-ITD positive patients ($p< 0.001$). A previous study[17] showed a significant difference in OS time ($p= 0.041$) and a marginal difference in EFS in CDA 79 variant (AC&CC) genotypes compared to the wild type and that DCK 360 variant genotype had significantly increased OS ($p= 0.006$) and EFS times ($p= 0.028$). This discrepancy between the Caucasian studies and this study may be attributed to the allele frequency differences between the Egyptians and Caucasians that might affect the clinical phenotype of AML.

We acknowledge the limitations of our study as no previous studies were done testing the impact of the studied SNPS on the outcome of AML patients treated with cytarabine based chemotherapy in the Egyptian population, so further studies are required on larger cohort of patients to confirm the impact of the studied SNPs. Also using deep profiling techniques as sequencing to cover a wider panel of SNPs, the cost was a limiting factor to apply this in our study.

In conclusion, the frequency of the variants reflected the specific ethnic make-up of our Egyptian AML patients with different geographical distribution. Studied SNPs genotypes had no significant influence on treatment response on day 28 of induction therapy. There was an increased risk of renal impairment in patients having the variant allele of DCK 201-T, while the risk of neurological toxicity was higher in patients bearing the variant allele of DCK 360-G,and the risk of liver impairment was higher in patients carrying the wild allele of CDA79-A The current study showed that hepatological, nephrological, neurological and hematological toxicities along with DCK 201C>T heterozygous CT genotype were the most significant prognostic factors for OS and DFS survival. Knowledge of the genetic variability of Ara C metabolizing genes (mainly DCK and CDA) could be critical in predicting disease outcome, cytarabine-induced toxicities, and survival, thus paving the way towards individualized therapy and improving the outcome of AML patients.

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