MICRORNAS AS PREDICTORS OF NUCLEOSIDE ANALOG SENSITIVITY IN ACUTE MYELOID LEUKEMIA

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Dedication

To Baba

My source of motivation and blessings.

Abstract

Acute myeloid leukemia (AML) is the most aggressive form of hematological malignancies. Despite advances in treating AML, development of resistance to nucleoside analog therapy remains one of the major obstacles in AML treatment. Various factors, including SNPs in genes, epigenetics, etc. play a role in mediating variability in response to nucleoside analog therapy. Recent studies have shown that microRNAs can also serve as regulators of gene expression that can contribute to variability in response to therapeutic agents. Thus the main objective of the thesis was to determine the role of microRNAs as predictors of variability in nucleoside analog response.

To our knowledge no studies have been reported that identify microRNAs as predictors of response to cytarabine therapy in AML patients. In chapter II we used a translational approach of conducting *in vitro* and clinical study to identify microRNAs that were predictive of overall survival in AML patients. Our study conclusively identified that miR107-Myb, miR-378-granzyme B and miR10a-MAP4K4 as miRNA-mRNA pairs that can be used as predictors of overall survival in AML patients. Additionally, we also showed that the miRNAs mechanistically regulate the expression of these mRNAs by binding to the 3'- untranslated region of these mRNAs. miRNAs can also cause variability in response to cytarabine therapy by regulating the expression of the genes involved in disposition of cytarabine. In chapter III we identified that miR-24 and miR-34a as regulator of DCTD (an enzyme involved in inactivation of cytarabine) and DCK (activating enzyme), respectively. These miRNAs along with other miRNAs can be used

as part of biomarker signature that can be used to predict the overall survival in AML patients.

In chapter IV, we determined the impact of cytarabine treatment on *in vivo* cytarabine-induced changes in leukemia cell transcriptome and miRNA expression, to evaluate their impact on clinical outcome. In the first part of this chapter, we identified key genes (such as tumor suppressors DKK3, TRIM33, PBRM1, an oncogene SET, cytidine-deaminase family members APOBEC2 and APOBEC3G) influenced by cytarabine infusion that were also predictive of response. In the second part of this chapter, using data from clinical studies, we identified several miRNAs that were altered by cytarabine treatment. The changes in the expression of these miRNAs resulted in alteration in gene expression that correlated with the overall survival in AML patients. Additionally, using cell lines we were able to identify various miRNA – mRNAs that were altered by drug treatment, indicating that the therapy itself can influence the predictive ability of miRNAs as biomarkers.

Significant data has been published on cytarabine as it is the standard of care in AML patients, however, there is limited knowledge about newer nucleoside analogs such as clofarabine. In chapter V, using *in vitro* methods, we identified several microRNAs, such as miR-16, miR-515 cluster, etc., that can be used as predictors of response for clofarabine therapy. Our data clearly suggests that there are several distinct microRNAs that can be used as predictors of response to clofarabine therapy in AML patients. We propose doing a clinical study to evaluate the clinical utility of these miRNAs as predictors of response.

In summary, using a translational approach, we were able to identify miRNAs and several miRNA-mRNA pairs that can be used as biomarkers of response to cytarabine therapy. Additionally, we also identified that drug therapy itself can influence the outcome in AML patients. These findings are clinically important as they will help provide a new strategy to optimize dosing of nucleoside analogs in AML patients which in turn would lead to better overall survival while reducing the side effects.

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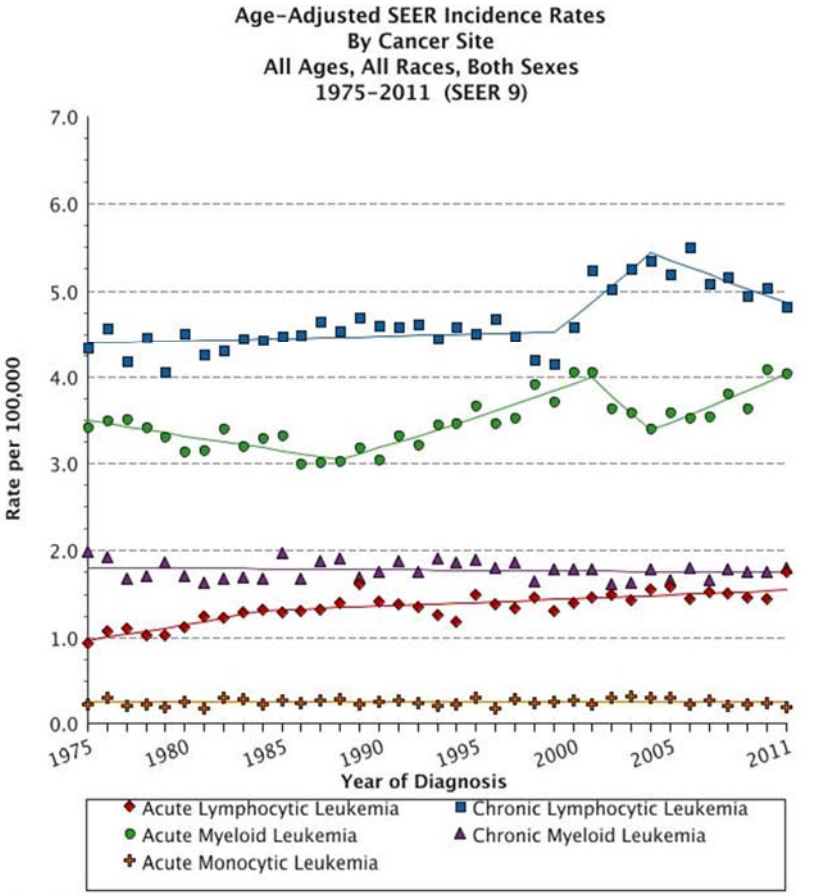
Literature review

1.1. Acute Myeloid Leukemia

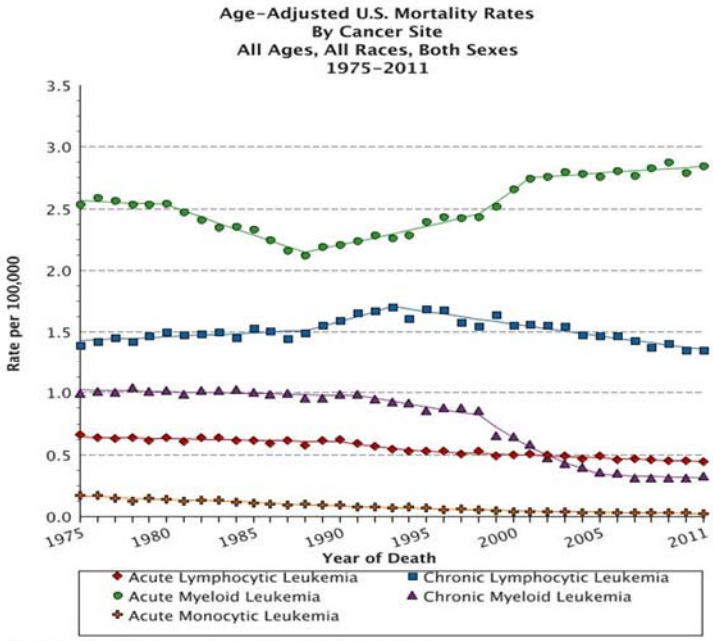
1.1.1. Introduction and Background: The word ‘leukemia’ is derived from the Greek words ‘leukos’ and ‘heima’ meaning ‘white blood’, which is mainly due to the initial observation of the abnormally high white blood cells in the patients, a characteristic feature of leukemia. Leukemias are broadly classified based on how quickly the disease progresses (acute and chronic) and the type of bone marrow cells that are affected (myeloid and lymphoid). The four most common types of leukemia are acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL). The other types of leukemias that do not strictly fall in the above categories include hairy cell leukemia, T-cell prolymphocytic leukemia, large granular lymphocytic leukemia, adult T-cell leukemia, etc.

Acute myeloid leukemia (AML) is a hematological malignancy, which results due to a series of genetic changes occurring in myeloid progenitor cells of the bone marrow. As a result of these malignant genetic transformations, there is an alteration of the normal hematopoietic growth and differentiation, resulting in accelerated production and accumulation of large numbers of poorly differentiated, immature and abnormal myeloid cells in the bone marrow and peripheral blood. Though these cells are capable of dividing and proliferating, they cannot differentiate into mature hematopoietic cells, disturbing the normal hematopoiesis process. Thus, AML mainly develops due to multiple genetic mutations leading to uncontrolled proliferation along with abnormal maturation.

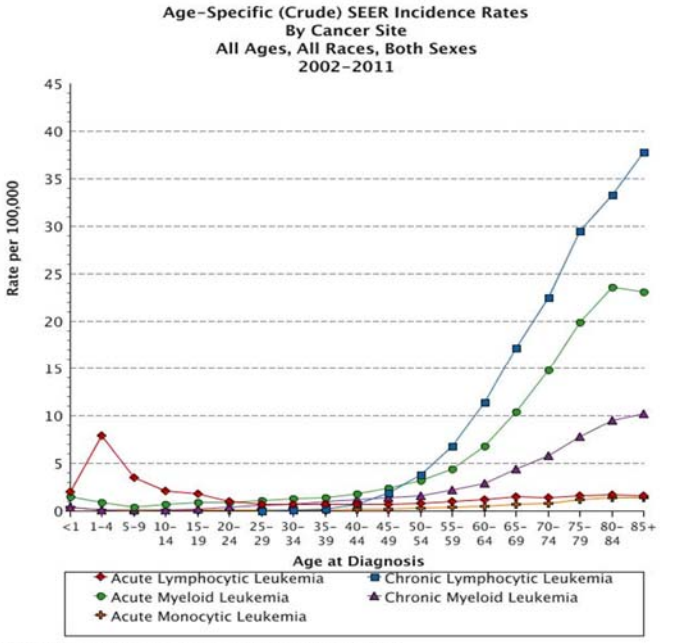
AML is the most common form of acute leukemia in adults with an incidence rate of 4.0501 cases per 100,000 patients. AML also has a highest mortality rate of 2.85 cases per 100,000 patients as compared to other leukemias.



Cancer sites include invasive cases only unless otherwise noted.
Rates are per 100,000 and are age-adjusted to the 2000 US Std Population (19 age groups - Census P25-1130). Regression lines are calculated using the Joinpoint Regression Program Version 4.1.0, April 2014, National Cancer Institute.
Incidence source: SEER 9 areas (San Francisco, Connecticut, Detroit, Hawaii, Iowa, New Mexico, Seattle, Utah, and Atlanta).



Cancer sites include invasive cases only unless otherwise noted.
Mortality source: US Mortality Files, National Center for Health Statistics, CDC.
Rates are per 100,000 and are age-adjusted to the 2000 US Std Population (19 age groups - Census P25-1130). Regression lines are calculated using the Joinpoint Regression Program Version 4.1.0, April 2014, National Cancer Institute.



Cancer sites include invasive cases only unless otherwise noted.
Rates are per 100,000.
Incidence source: SEER 18 areas (San Francisco, Connecticut, Detroit, Hawaii, Iowa, New Mexico, Seattle, Utah, Atlanta, San Jose-Monterey, Los Angeles, Alaska Native Registry, Rural Georgia, California excluding SF/SJM/LA, Kentucky, Louisiana, New Jersey and Georgia excluding ATL/RG).
Datapoints were not shown for rates that were based on less than 16 cases.

Figure 1.1: Prevalence and Incidence of AML

According to the Cancer Facts & Figures published by the American Cancer Society (ACS), the prevalence of AML is 35,726, as of January 2010. It is estimated that around 18,860 new AML cases in both adults and children would be diagnosed in the US in 2014 and approximately 10,460 deaths would be attributed to AML. It was reported that the 5-year relative survival rates from 2003 to 2009, for AML were 24.9% overall and 64.8% children and adolescents younger than 15 years (Figure 1.1). Although AML can occur at any age, the median age for diagnosis of AML is mainly around 69 years (SEER statistics). Even though AML is not common in children and adolescents, AML constitutes around 20% of the childhood acute leukemias [1].

1.1.2. Classification of AML: AML mainly refers to the malignancies originating from the myelocytes or granulocytes, but could also be applied to leukemias of myeloid, monocytic, erythroid, or megakaryocytic origin. Hence, AML is considered to be a heterogeneous disease, which includes several subtypes with different morphology, immunophenotype and cytogenetic characteristics. Classification of AML into different subtypes helps decide the prognosis and guide the treatment regimen. The first classification of AML was proposed by French-American-British (FAB) co-operative group in 1976 and revised in 1985 and 1991, and was largely based on the cytomorphological characteristics of the cells [2-7]. The FAB classification divides AML into eight subtypes depending mostly on how the leukemia cells lineage and the maturity of the cells (Table 1.1) [5].

The FAB classification of AML was the first to provide a uniform approach to classify AML and is still used to stratify AML patients into different subtypes. However, the FAB

classification failed to take into account the underlying genetic abnormalities and immunologic characteristics that are generally associated with AML.

Table 1.1: French-American-British (FAB) Classification of AML

FAB Subtype	Morphology
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Hypergranular acute promyelocytic leukemia
M3 variant	Hypogranular variant acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M4 eos	Acute myelomonocytic leukemia with dysplastic eosinophilia
M5a	Acute monoblastic leukemia
M5b	Acute monocytic leukemia
M6	Erythroleukemia
M7	Megakaryoblastic leukemia

However the morphologic, immunologic and cytogenetic (MIC) classification, which was introduced by the MIC Cooperative Study Group in 1986 [8-10] tried to overcome this shortcoming and took into consideration the importance of cytogenetic characteristics of AML, along with the morphology and immunology of the disease, for its classification. This MIC classification provided additional insight into the clinical significance of cytogenetics for AML classification, which is depicted in the Table 1.2 [10, 11].

Table 1.2: Morphologic, Immunologic and Cytogenetic (MIC) Classification of AML

		Immunologic markers						
MIC group	**FAB**	**CD34**	**CD33**	**CD13**	**CD11**	**CD15**	**CD14**	**Karyotype**
M2 / t (8; 21)	M2	+/-	+	+	+	+	+/-	t (8; 21) (q22;q22)
M3 / t (15; 17)	M3, M3v	–	+	+	+/-	+/-	-	t (15; 17) (q22;q12)
M5a / del (11q23)	M5a (M5b, M4)	+/-	+	+	+	+	+	t /del(11) (q23)
M4Eo / inv (16)	M4Eo	+/-	+	+	+	+	+	del / inv(16)(q23)
M1 / t (9; 22)	M1 (M2)	+	+	+	+/-	+/-	+/-	t (9;22)q(34;q11)
M2 / t (6; 9)	M2/M4 with basophilia	+/-	+	+	+	+	+/-	t (6;9)(p21-22;q34)
M1 / inv (3)	M1 (M2, M4, M7) with thrombocytosis	+/-	+	+	+/-	+/-	+/-	inv (3)(q21q26)
M5b / t (8;16)	M5b with phagocytosis	+/-	+	+	+	+	+	t (8;16)(p11;p13)
M2 Baso / t (12p)	M2 with basophilia	+/-	+	+	+	+	+/-	t /del(12)(p11-13)
M4/+4	M4 (M2)	+/-	+	+	+	+	+/-	+4

The World Health Organization (WHO) proposed another method of classification of AML in 2001. WHO classification is based on the combination of morphological features, clinical syndrome, immunophenotype, cytogenetic and other biological features of AML [11, 12]. Table 1.3 lists the different groups in to which AML is classified based on WHO classification.

Table 1.3: Classification of AML based on WHO guidelines

Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
APL with t(15;17)(q22;q12); PML-RARA
AML with t(9;11)(p22;q23); MLLT3-MLL
AML with t(6:9)(p23;q34); DEK-NUP214
AML with inv(3)(q21q26.2) or t(3.3)(q21;q26.2); RPN1-EVl1
AML (megakaryoblastic) with t(1:22)(p13;q13); RBM15-MKL1
AML with mutated NPM1
AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified (NOS)
AML with minimal differentiation
AML without maturation
AML with maturation

Acute myelomonocytic leukemia
Acute monoblastic and monocytic leukemia
Acute erythroid leukemia
Acute Megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down's syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia with t(9;22)q(34;q11.2); BCR-ABL1
Mixed phenotype acute leukemia with t(v;11q23); MLL rearranged
Mixed phenotype acute leukemia, B/myeloid, NOS
Mixed phenotype acute leukemia, T/myeloid, NOS
Natural killer (NK) cell lymphoblastic leukemia/lymphoma

1.1.3. Prognosis of AML: The major prognostic factors related to AML are mainly to predict the treatment related mortality and resistance to chemotherapy [13, 14]. The prognosis of AML depends on both patient-related factors and disease-related conditions.

The important patient-related factors that predict the treatment related mortality are age, organ function of the patient and performance status. According to the Surveillance, Epidemiology and End Results database (SEER) it is estimated that AML is more frequently diagnosed in elderly patients, with a median age of diagnosis being 69 years in the US population. Also, older patients have worse treatment outcomes as compared to younger patients diagnosed with AML. In addition to age being an independent predictor of poor prognosis, older patients also present with additional factors such as comorbidities, adverse cytogenetic abnormalities and poor performance status [15]. The prognostic factors such as cytogenetics, molecular abnormalities, and minimal residual disease mainly predict resistance to chemotherapy. Cytogenetic abnormalities have been strong predictors of the clinical outcomes in both pediatric and adult AML patients [16]. Based on cytogenetic features, AML is divided into three risk distinctive risk groups, which are used to design treatment strategies, and predict clinical response. Three distinct risk groups of AML include favorable, intermediate and adverse. Many genetic studies have also shown the significance of genetic mutations on the clinical response in both pediatric and adult leukemias. These genetic mutations were detected in AML patients especially with normal karyotype. The standardized stratification of AML was proposed by European Leukemia Net (ELN) taking into account both cytogenetic and molecular genetic data [14].

Table 1.4: Classification of patients based on Cytogenetics

Prognostic risk group	Cytogenetic abnormality and affected gene
Favorable	t(8;21)(q22;q22); RUNX1RUNX1T1
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD (normal karyotype)
	Mutated CEBPA (normal karyotype)
Intermediate-I*	Mutated NPM1 and FLT3-ITD (normal karyotype)
	Wild-type NPM1 and FLT3-ITD (normal karyotype)
	Wild-type NPM1 and without FLT3-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); MLLT3-MLL
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
	t(6;9)(p23;q34); DEK-NUP214
	t(v;11)(v;q23); MLL rearranged
	-5 or del(5q); -7; abnl(17p); complex karyotype

*Includes all AMLs with normal karyotype except for those included in the favorable subgroup; most of these cases are associated with poor prognosis, but they should be reported separately because of the potential different response to treatment.

1.1.4. Treatment of AML: There are various treatment options available for AML and the choice of treatment depends on the molecular subtype of the disease, the cytogenetics of the leukemic cells and also the age of the patient [17, 18]. These factors are important to be taken under consideration since they can influence the responsiveness to chemotherapy and the risk of relapse of the disease. The treatment of AML is primarily divided into three phases – remission induction (or induction) chemotherapy, post-remission (or consolidation) chemotherapy and maintenance chemotherapy (Figure 1.2). The primary goal of the induction therapy is to achieve complete remission, which is defined as ≤5 % blasts in bone marrow. The subsequent more intensive consolidation therapy aims at destroying the residual leukemic blasts and preventing relapse of the disease.

The induction treatment of AML takes into account the age and cytogenetics of the patients [15]. The induction therapy for older patients (greater than 60 years of age) with good performance status, minimal comorbidities, and favorable cytogenetics involves continuous infusion of standard dose of cytarabine (100-200 mg/m^2) for 7 days along with 3 days of anthracycline. Older patients who cannot undergo intensive chemotherapy are usually given low dose cytarabine.

In younger patients, remission induction therapy includes cytarabine given in combination with an anthracycline, like daunorubicin or idarubicin. The standard of care for the induction treatment usually consists of a combination of 3 days of daunorubicin and 7 days of cytarabine (3+7). Cytarabine is given in a daily standard dose of 200 mg/m^2 by continuous infusion or twice per day bolus or high dose of 2-3 gm/m^2. Even though

cytarabine-based induction regimens form the backbone of AML treatment, around 20 to 45% patients do not achieve complete remission (CR) with standard induction chemotherapy and the majority of the patients who achieve CR are known to relapse [19-21]. If residual blasts are detected in bone marrow of patients, standard-dose cytarabine or high-dose cytarabine with anthracycline is the most common salvage therapy. Consolidation treatment usually consists of multiple cycles of high dose cytarabine followed by maintenance therapy.

Maintenance chemotherapy is usually considered to be less myleosuppressive than induction or consolidation thermotherapy [22, 23]. The primary goal of starting AML patient on maintenance chemotherapy is to further reduce the leukemic cell burden and prevent relapse. [24-26]. However the significance and benefits of maintenance chemotherapy in the treatment of AML is thought to be controversial and depends on the intensity of the induction and consolidation chemotherapies [20, 25].

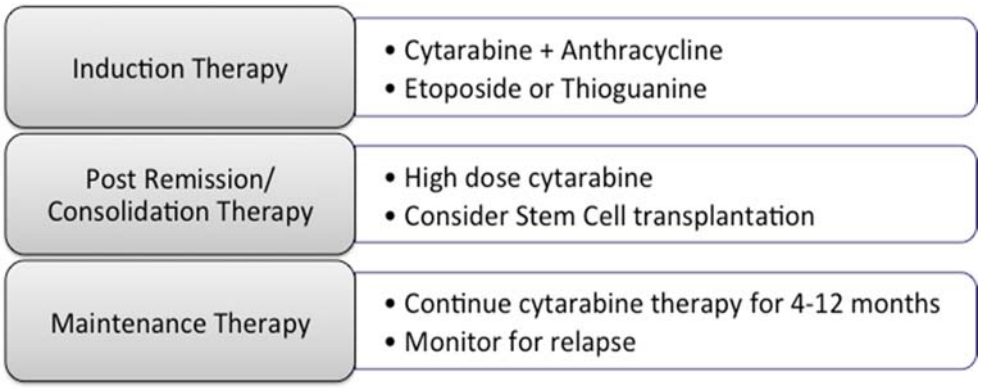


Figure: 1.2: Overall Dosing Strategy in AML patients

In order to further optimize AML chemotherapy, newer cytotoxic drugs and targeted therapies are being explored which could be incorporated into the current chemotherapy

regimens, since further dose intensification of the current treatment is unlikely to improve clinical outcome [27-29]. Hypomethylating agents 5-azacitidine or decitabine have been used as induction remission therapy for older patients [30-33]. Other nucleoside analogs like clofarabine, cladribine, fludarabine, troxacitabine and sapacitabine have also been investigated in various induction regimens for AML patients [34-40]. Clofarabine, which is approved by FDA for relapsed pediatric, ALL, has shown promise as a single agent therapy for older patients in phase II clinical trial with overall remission rate of 46% and a 30-day mortality rate of 10% [41] as well as in combination with cytarabine [42]. Apart from the nucleoside analogs, other targeted therapies for AML include antibody directed chemotherapy with gemtuzumab ozogamicin, a humanized anti-CD33 antibody) [43-46] and lintuzumab, a humanized monoclonal antibody with a human immunoglobin G1 framework [47, 48], are also being investigated to improve therapy for AML patients. The section below (Section 1.2) provides an overview of the two of the most important chemotherapy drugs used for the treatment of AML, namely cytarabine and clofarabine (broadly classified as nucleoside analogs).

1.2. Nucleoside Analogs

Nucleoside analogs are a family of anti-metabolites that resemble the endogenous nucleosides. Nucleoside analogs are extensively used as antiviral drugs as well as anticancer agents. As anticancer chemotherapeutic agents, nucleoside analogs are widely used for the treatment of solid tumors, hematological malignancies, and autoimmune disorders. Cytarabine is the most commonly used nucleoside analog for the treatment of

AML while, clofarabine is a relatively newer nucleoside analog that is still under investigation in various clinical trials in patients with AML.

1.2.1. Cytarabine: Cytarabine (1-β-D-arabinofuranosylcytosine), also known as cytosine arabinoside or ara-C, is an analog of deoxycytidine, which forms the backbone of AML treatment. Cytarabine differs from the physiological nucleoside by the presence of a hydroxyl group in the β-configuration at the 2'-position of the ribose sugar (Figure 1.3).

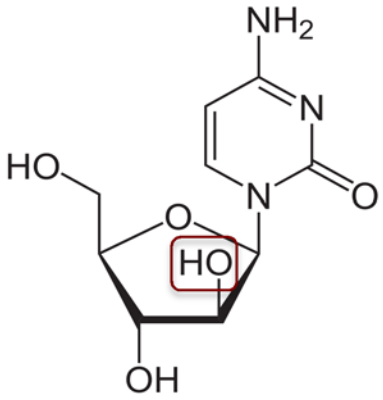


Figure 1.3: **Cytarabine**

Standard dose cytarabine (100-200 mg/m^2) achieves steady-state plasma levels of 0.5 -1 μM [49, 50]. At this concentration the expression of equilibrative nucleoside transporter (hENT1), which is the influx transporter involved in the cellular uptake of cytarabine, is the rate-limiting factor in its uptake. With high dose cytarabine (2-3 gm/m^2) plasma concentrations greater than 50 μM are achieved and at these concentrations, simple diffusion rates exceed the carrier-mediated transport [51, 52]. Once inside the cell cytarabine is phosphorylated by deoxycytidine kinase (dCK) to cytarabine monophosphate, which is rate limiting step in the phosphorylation of cytarabine (Figure

1.4). Cytarabine monophosphate is further phosphorylated by cytidine monophosphate kinase (CMPK) and nucleoside diphosphate kinase to the diphosphate and the active, triphosphate form of cytarabine. Cytarabine is rapidly metabolized by cytidine deaminase (CDA) to its biologically inactive metabolite, 1-β-D-arabinofuranosyluracil (arabinoside uridine), while the monophosphate form of cytarabine is deaminated by the action of deoxycytidylate deaminase (DCTD). The 5'-nucleotidase enzymes (NT5C2/3) are responsible for dephosphorylation of cytarabine, thus opposing the action of dCK. Cytarabine triphosphate acts as an inhibitor of DNA polymerase-α as well as it also serves as a substrate for this enzyme and competes with deoxycytidine triphosphate (dCTP) for incorporation into the growing DNA strand (Figure 1.4) [53-55]. The triphosphate form of cytarabine also inhibits DNA polymerase-β and subsequently inhibits DNA repair [56-58]. Cytarabine cytotoxicity is mainly due to the combination of inhibition of DNA polymerase and from incorporation of the cytarabine triphosphate into the growing DNA strand, in competition with endogenous deoxycytidine triphosphate (dCTP). This ultimately leads to chain termination of the DNA strand inhibiting DNA synthesis [54, 59, 60]. Both the concentration and duration of exposure of active triphosphate metabolite of cytarabine are critical for the mechanism of action of this nucleoside analog [61]. Typically cytarabine is administered as standard low dose of 100mg/m^2/day continuous IV infusion for 7 days or 100mg/m^2 IV every 12 hours for 7 days, or as high dose of 2-3 gm/m^2 IV infusion for over 1-3 hours every 12 hours for 2-6 days. The steady state plasma concentrations achieved after standard low dose cytarabine are 0.5-1μM, while high dose cytarabine achieves steady state plasma concentrations of

greater than 10μM. Activity of cytarabine is primarily decreased by its rapid deamination by CDA to biologically inactive metabolite, 1-β-D-arabinofuranosyluracil and greater than 80% of the drug undergoes renal excretion. Systemic elimination of cytarabine is biphasic, with initial plasma half-life ($t_{1/2}$ α) of 7 to 20 minutes and terminal half-life ($t_{1/2}$ β) of 2 to 3 hours [61-63].

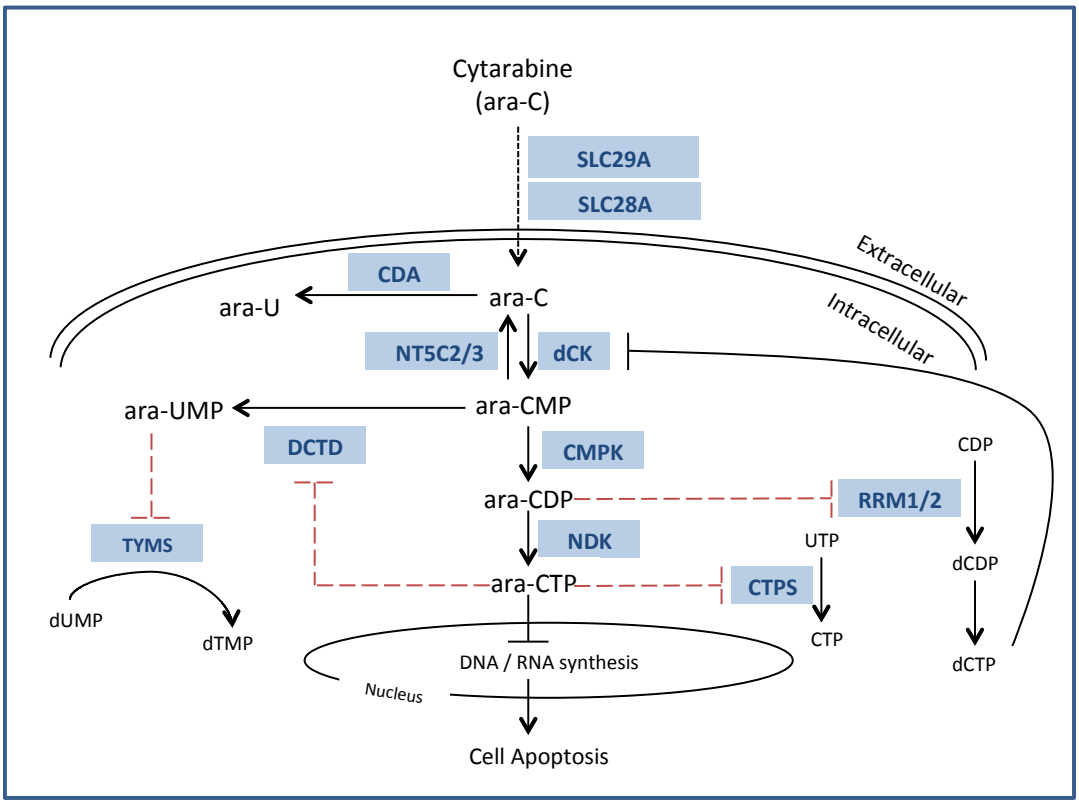


Figure 1.4: PK/PD pathway of Cytarabine

1.2.2 Clofarabine: Clofarabine (CAFdA, 2-chloror-9-(2'-deoxy-2'-fluoro-D-arabinofuranosyl) adenine is a second generation purine nucleoside analog which was designed as a hybrid molecule to overcome the limitations of the first generation purine analogs, cladribine and fludarabine (Figure 1.5) [64, 65]. Similar to other purine

nucleoside analogs, clofarabine is transported into the cell by equilibrative nucleoside transporters, hENT1 and hENT2, as well as concentrative nucleoside transporter hCNT3 [64]. Like cytarabine, clofarabine needs to be converted to its active triphosphate metabolite by various enzymes. Conversion of clofarabine monophosphate to diphosphate metabolite is believed to be the rate-limiting step in the activation of this purine analog [66].

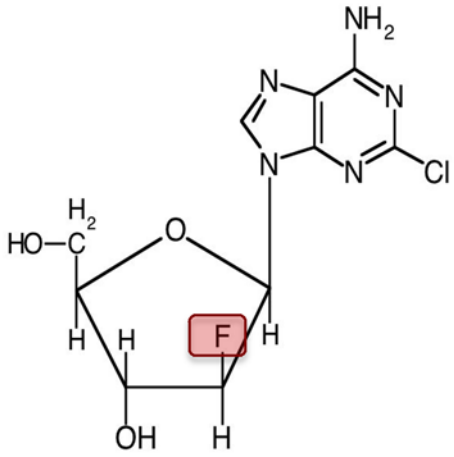


Figure 1.5: **Clofarabine**

Clofarabine triphosphate is known to inhibit both DNA polymerases and ribonucleotide reductases, thus inhibiting DNA synthesis and repair [67]. Clofarabine also induces apoptosis through induction of DNA strand breaks and disruption of mitochondrial integrity, resulting in release of proapoptotic proteins [68]. Clofarabine is currently approved for the treatment of relapsed or refractory acute lymphoblastic leukemia (ALL). Clofarabine has also been studied as a single agent or a combination agent with cytarabine for the treatment of AML. A study in older AML patients with an induction regimen consisting of clofarabine and high dose cytarabine showed promising results with good response rate (overall response rate of 60%) and with an acceptable toxicity

profile [69]. Another study compared the effects of clofarabine plus cytarabine with cytarabine alone in patients ≥55 years old with refractory or relapsed AML [70]. The response rate and event free survival (EFS) was significantly improved in the clofarabine plus cytarabine arm compared to the cytarabine alone arm, although there was no difference in the overall survival (OS) between the two arms. For AML, clofarabine monotherapy is usually 20mg/m^2 IV once daily for 5 days, with cycles repeated every 4 to 6 weeks [71] or salvage therapy includes 40mg/m^2/day IV over 1 hour for 5 consecutive days every 3 to 6 weeks [72].

The cellular elimination kinetics of the phosphorylated clofarabine metabolites shows triphasic kinetics with terminal half-life ($t_{1/2}$ β) of 8 to 24 hours and a very long γ half-life (>24 hours) indicating prolonged cellular retention [66, 73].

1.3. Factors affecting activation of Nucleoside Analogs and Clinical Response in AML patients:

Nucleoside analogs are transported into the cells by nucleoside transporters and are converted to their active metabolites by the action of various enzymes. The most useful parameter for predicting the efficacy of nucleoside analogs, apart from its plasma concentrations, is the active triphosphate levels in the leukemic cells [74-77]. *In vitro* studies by Kufe DW et al. have shown that leukemic cells resistant to cytarabine have lower levels of active triphosphate metabolite of the drug as compared to the sensitive cells [78]. In addition, in a separate clinical study, higher cytarabine triphosphate levels were observed in patients with AML who were responsive to the cytarabine treatment as

compared to the CML patients who were not responsive to this treatment [79]. Apart from predicting the sensitivity of the treatment, intracellular triphosphate levels for cytarabine have also been associated with clinical response with high-dose cytarabine treatment [74]. Evidence has shown that serious toxicities and side effects, such as myelosuppression, thrombocytopenia, cardiac toxicity, etc., are also related to the active phosphorylated metabolites inside the leukemic cells [80, 81].

Thus, it is obvious that with respect to the nucleoside analogs the intracellular levels of the active phosphorylated metabolites are crucial for predicting the efficacy and clinical outcome. The primary factors that regulate the intracellular triphosphate levels are the levels/activity of the nucleoside transporters, levels of the activating (kinases) and inactivating enzymes and the intracellular deoxynucleotide pools, which are regulated by ribonucleotide reductases. Factors that could affect the expression of these transporters and enzymes, could in turn potentially affect the levels of the active metabolite of nucleoside analogs. Some of the important factors that might potentially affect the expression and/or activity of nucleoside analog pathway genes are inherited genetic polymorphisms, acquired somatic mutations, regulation or altered regulation by microRNAs, inter-patient differences in methylation levels as well as a much less studied aspect of drug-induced changes in the expression of the genes.

1.3.1. Genetic Polymorphisms: Single nucleotide polymorphisms (SNPs) in the genes encoding the drug transporters, activating and inactivating enzymes as well as the pharmacodynamic (PD) targets of these drugs can influence the clinical outcome of the patients treated with these nucleoside analogs. SNPs and small insertions or deletions in

these genes could result in amino acid changes that could in turn influence the protein and/or the activity of the respective genes. Presence of SNPs in the regulatory regions of these genes can have an effect on the transcription of the mRNA, leading to changes in the gene expression levels [82].

Genetic variation in the key enzymes and transporters involved in the metabolic pathway of nucleoside analogs are described below and provided in table 1.5. Variability in expression of human equilibrative nucleoside analog transporter (hENT1) has been associated with differential transcriptional regulation, with SNPs in the proximal promoter region (-1345 C>G, -1050 G>A, -706 G>C) associated with loss of binding sites for transcription factors [83]. The phosphorylation of nucleoside analogs by DCK being the rate-limiting step in the activation of nucleoside analogs, the expression of this kinase is important with respect to the outcome of AML patients. Studies have shown that variability in treatment outcome in AML patients treated with cytarabine correlated with DCK mRNA expression [84]. The regulatory SNPs (−360 C>G and −201 C>T) in the promoter of DCK were found to be associated with the mRNA expression of DCK [85]. Sequencing the DCK promoter and coding exons identified three nonsynonymous SNPs (Ile24Val, Ala119Gly and Pro122Ser); the variant forms of which demonstrated reduced DCK activity [86]. A SNP in the 3' untranslated region (UTR) of DCK (rs4643786) was found to be associated with DCK mRNA expression in both European and African ancestry panels of cell lines as well as with lower leukemic blast cell intracellular levels of cytarabine triphosphate [86]. Similarly, various other studies have reported the significance of SNPs in other important nucleoside pathway genes, like

NT5C2, CDA, DCTD and RRM1/2 with respect to nucleoside analogs cytotoxicity and /or response [87-92].

The table below (Table 1.5) provides an overview of the studies by our group and others on various genetic polymorphisms in cytarabine genes and their relevance in AML.

Table 1.5: Important single nucleotide polymorphisms (SNPs) in the nucleoside analog metabolic pathway genes

Gene	Gene symbol	SNPs	Relevance	PMID
Deoxycytidine kinase	DCK	rs2306744	SNPs -360C>G and -201C>T (rs2306744) in DCK were associated with higher mRNA expression and higher transcriptional activity. In AML patients, it was found that -360G/-201T haplotype was associated with favorable response.	15564883
Deoxycytidine kinase	DCK	rs4694362	In Korean AML patients, the CC genotype was a significant poor prognostic factor for poor overall survival.	22964418
Deoxycytidine kinase	DCK	rs111454937	AA genotype was more frequent in Chinese AML patients with higher platelet count and A allele frequency was significantly higher in the group <40 years, lower WBC count patients group and group with platelet count >60×109/L.	22884143
Deoxycytidine kinase	DCK	rs72552079	In Chinese AML patients, the TC genotype showed significantly improved Ara-C based chemotherapy response.	22884143
Deoxycytidine kinase	DCK	rs11554389	In Chinese AML patients, the TC genotype showed significantly improved Ara-C based chemotherapy response.	22884143

Cytidine deaminase	CDA	rs2072671 rs532545	In FLT3-ITD positive AML patients, the CDA 79C/C (rs2072671) and CDA -451T/T (rs532545) genotypes were associated with shorter overall survival compared to other genotypes.	23873772
Cytidine deaminase	CDA	rs2072671	Plasma CDA activity was significantly decreased in samples homozygous for the A79C SNP (CC) compared with samples homozygous for the ancestral allele (AA).	23287564
Cytidine deaminase	CDA	rs60369023	Significantly improved Ara-C based chemotherapy response in Chinese AML patients.	22884143
Cytidine deaminase	CDA	rs3215400	In AML patients, there was a significantly higher CDA RNA expression in patients homozygous for -33/-31 delC mutant as compared with those with heterozygous or wild type genotypes.	22304580
Cytidine deaminase	CDA	rs75720390	AML patients with intron 2 (TCAT) 5/5 and (TCAT) 5/4 genotypes showed significantly higher CDA expression as compared to those with wild type (TCAT) 4/4 repeats.	22304580
Cytidine deaminase	CDA	rs71864371 rs1048977	The 3'-UTR 816 in/delC variants in LD with synonymous coding SNP 435C>T in exon 4 was found to be significantly associated with lower CDA RNA expression compared to the wild type in AM patients.	22304580

Cytidine deaminase	CDA	rs532545	AML patients with the CDA C-451T/promoter SNP TT-genotype had significantly higher lactate dehydrogenase levels when compared with the CC or CT-genotypes (P=0.01).	19458626
Cytidine deaminase	CDA	rs2072671 rs602950	AML patients with AA genotype for CDA A79C SNP and AA genotype for CDA A-92G SNP had lower incidences for grade III/IV liver toxicity when compared with the other respective genotypes.	19458626
Nucleoside diphosphate kinase 1	NME1	rs2302254	Caucasian AML patients with the T_T genotype for promoter SNP -835C/T had a significantly lower platelet count and better ECOG performance status compared to the patients with C_C genotype. This study also identified an increased risk of neurotoxicity for T_T genotype.	22035418
Ribonucleotide reductase M1	RRM1	rs1042919 rs1561876	In AML patients receiving cytarabine and cladribine, these SNPs are associated with intracellular CTP level, response after remission induction therapy, risk of relapse and overall survival.	24024897
Solute carrier family 29 (nucleoside transporters), member 1	SLC29A1	rs3734703	In AML patients, the AA and AC genotype for SLC29A1 SNP rs3734703 in combination with TYMS rs2612100 (AA genotype) was found to be significantly associated with shorter relapse free survival.	22964418

ATP-binding cassette, sub-family C, member 3	ABCC3	rs4148405	The SNP rs4148405 was found to be associated with significantly shorter disease free survival in AML patients, with the minor allele (G) being associated with shorter time to relapse.	23677058
ATP-binding cassette, sub-family B, member 1	ABCB1	rs1128503 rs2032582	AML patients with 1236 C/C genotype for SNP rs1128503 or 2677 G/G genotype for SNP rs2032582 showed poorer survival as compared to patients with other genotypes for that the respective SNPs.	20938465

* Table from Book Chapter- Pharmacogenetic Studies in Pediatric Acute Myeloid Leukemia. Book Title: Genome-Wide Association Studies: From Polymorphism to Personalized Medicine. (Cambridge University Press) Bhise NS, Chauhan L, Lamba JK.

1.3.2. MicroRNAs – Introduction and Discovery: MicroRNAs (miRs) are endogenous, single stranded, small noncoding RNAs which are about 22 nucleotides in length that regulate gene expression by binding to specific mRNA target sequences and promoting their degradation or translational repression. Various bioinformatics predictions estimate that microRNAs are known to regulate approximately 30% to 60% of the human protein coding genes [93-95]. Various studies have implicated a role of microRNAs in many biological process and molecular functions [96]**.** The discovery of the effects of lin-4 RNA and its effect on the lin-14 gene led to the discovery of microRNAs. In 1993, the joint efforts of Victor Ambrose and Gary Ruvkun laboratories discovered that, in *C.elegans*, small 22 nucleotide RNA called *lin-4*, which does not encode for any protein, had antisense complementarity to 3'UTR sites of *lin-14* gene. However, in 2000 another short noncoding RNA, *let-7* was found to be conserved across various species. This discovery drew the interest of other researchers in these small noncoding RNAs. The discovery of microRNAs added another dimension to the central dogma of molecular biology, that DNA is transcribed into RNA which is then translated into proteins which then carry out various biological functions. The discovery of microRNAs changed this understanding that RNA molecules are mere 'secondary players' by transcribing information from DNA, since microRNAs can in fact regulate the gene expression too.

1.3.2.1. MicroRNA Biogenesis: MicroRNA genes are encoded in the intergenic regions of the genome or in the annotated transcripts, such as introns of protein coding genes, introns of noncoding genes or exons on noncoding genes [97-99]. The

transcription of most of the primary transcripts of the microRNAs is mainly by polymerase II (pol II) and while polymerase III (pol III) is known to play a role to a lesser extent (Figure 1.6) [99]. The primary transcripts of the microRNAs are referred to as primary precursors of miRNA or pri-miRNAs [100]. These pri-miRNAs are large stem-loop structures, containing 7-methylguanosine cap and a poly (A) tail [98, 99]. Inside the nucleus, the pri-miRNAs are cleaved into 70- to 100-nucleotide hairpin shaped microRNA precursors or pre-miRNAs by nuclear microprocessor protein complex formed by RNase III enzyme Drosha (RNASEN) and DGCR8 (DiGeorge syndrome critical region 8), also known as Pasha-partner of Drosha (Figure 1.6) [101-103]. Following nuclear processing by Drosha, the pre-miRNAs are then exported in the cytoplasm by Exportin-5 and co-factor, Ran guanosine triphosphate (Ran-GTP) complex [104, 105]. In the cytoplasm, the pre-miRNAs then undergo further processing by another RNase III endonuclease enzyme, Dicer [106]. The Dicer cleaves the pre-miRNAs into about 22- to 24-nucleotide miRNA duplex, leaving the 5' phosphate and a 2-nucleotide overhang (Figure 1.6). The double stranded RNA-binding domain proteins TRBP (Tar RNA binding protein) and PACT (protein activator of PKR) facilitate the Dicer mediated cleavage of pre-miRNA [107, 108]. This microRNA duplex produced by Dicer comprises of one mature miRNA strand or guide strand and another similar sized fragment derived from the opposite strand, called as miRNA* or passenger strand [109]. In this miRNA-miRNA* duplex, the strand with the less stable base pairing at its 5' end in the duplex is selected as the guide strand and loaded onto Argonaute (Ago) proteins to form RNA-induced silencing complex (RISC) [110, 111]. The guide strand of the miRNA-miRNA*

duplex is believed to undergo rapid degradation. However some reports have indicated that the miRNA* strand could also loaded into the RISC and be functional [112].

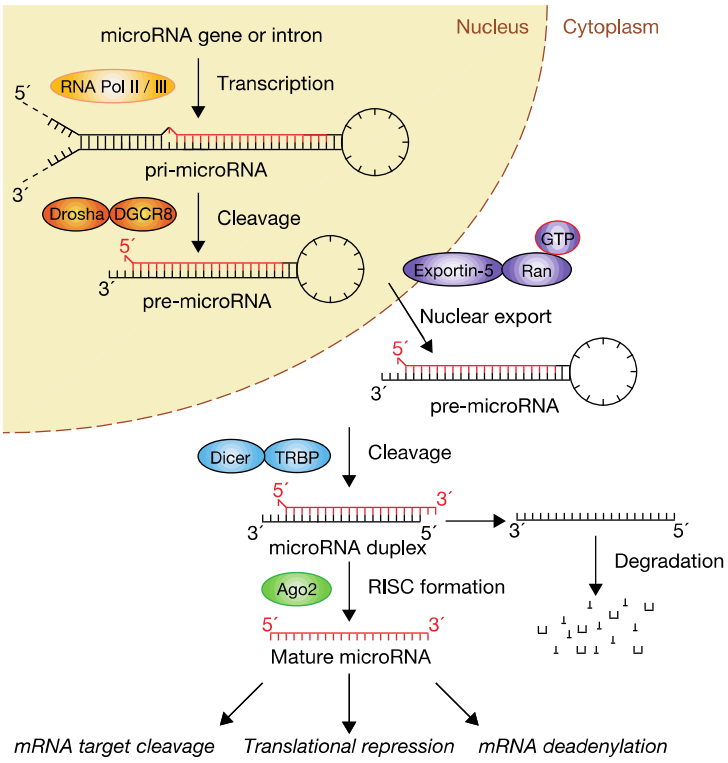


Figure 1.6: Biogenesis of microRNAs (Adapted from Winter J, 2009)

1.3.2.2. MicroRNA function and their role in Cancer

MicroRNAs have been extensively studied with regards to various diseases since their discovery. Specifically, the understanding of the influence of microRNAs in pathogenesis of various human cancers has increased. The first report implicating the role of microRNAs in cancer, specifically chronic lymphocytic leukemia (CLL) was in 2002 [113]. This study reported that miR-15a and miR-16-1 are located at chromosome 13q14, a region frequently deleted in CLL, thus suggesting an association between these microRNAs and CLL. Thereafter the same group identified a significant number of

microRNAs located at fragile sites and in regions altered in cancers, including regions of amplification or loss of heterozygosity or break points, identifying microRNAs as new class of regulators in human cancers [114]. Many studies have also reported disease specific expression profiles with important diagnostic and prognostic implications in various cancers, like B cell CLL [115], breast cancer [116], primary glioblastoma [117], hepatocellular carcinoma [118], gastric and colon cancers [119], papillary thyroid carcinoma [120], and endocrine pancreatic tumors [121]. With availability of various microRNA expression profiling techniques, many researchers have identified specific microRNAs up- or down-regulated in various cancers relative to normal tissues [122, 123]. In addition, microRNA expression profiling has also been used to classify large panel of tumor types based on their global microRNA expression levels. These researcher believe that this system of classification is of significant clinical importance in predicting cancer type/stage as compared to classification based on mRNA expression profile [123]. MicroRNAs have also been implicated to play an important biological functional role in the development of cancer. MicroRNAs have been shown to act as oncogenes (oncomirs) or tumor suppressors and are involved in various pathways that are specifically deregulated in various cancers [124]. The first reports addressing the biological role of microRNAs in cancer was demonstrated by genetic studies in *C.elegans* where the authors showed that let-7 family member, miR-84 negatively regulates the expression of let-60, an ortholog of human oncogene RAS [125]. Similar to their *C. elegans* ortholog, the human KRAS and NRAS oncogenes were found to be regulated by human let-7 family members. Accordingly, in lung tumor samples the decreased expression of

microRNA let-7 correlated with increased protein levels of RAS oncogene, explaining the functional significance of let-7 as tumor suppressor [125]. The tumor suppressor role of microRNAs miR-15a and miR-16-1, which were frequently found to have low expression in CLL, appears to be partly mediated by their ability to decrease the expression of anti-apoptotic protein Bcl2 found to be overexpressed in CLL promoting cell survival [126, 127]. The expression of these microRNAs was found to promote apoptosis in leukemia cell lines, which explains that the loss of function of miR-15a and miR-16-1 in CLL leads to increase Bcl2 expression and prevents cell apoptosis. The oncogenic role of microRNAs is evidenced by studying the miR-17 cluster encoded in a region frequently amplified in B-cell lymphomas [128]. The increased expression of miR-17 cluster in B-cell lymphoma mouse models expressing c-myc transgene, lead to increased lymphomagenesis [129]. Similarly, many such microRNAs have been studied and implicated to play an important role in various cancers. These and other studies provide evidence that miRNA deregulation is not just a consequence of the malignant transformation, which results in loss of normal cellular identity, but in fact miRNAs have unique oncogenic and tumor suppressor roles which are critical in pathogenesis of cancer.

1.3.2.3. Role of MicroRNAs in Acute Myeloid Leukemia

Various miR-expression profiling and functional studies have implicated that microRNAs play an important role in regulation of normal hematopoietic process [130, 131]. Many studies have shown that deregulated expression of these microRNAs can contribute to leukemogenesis. The expression of miR-181 was found to be higher in B cells and induction of expression of this microRNA in hematopoietic stem cells stimulated their

differentiation into B-lineage cells [132]. Another microRNA, miR-328 was found to stimulate the differentiation of myeloid cells by promoting the expression of C/EBPα, a master regulatory transcription factor [133]. On the other hand, inhibition of C/EBPα mRNA and protein expression by miR-124a inhibits the differentiation of the cells toward the myeloid lineage [134].

In addition, microRNA expression signatures have been shown to distinguish ALL patients from AML patients, with 27 differentially expressed microRNAs identified between ALL and AML [135]. Among the most significantly differentially expressed microRNAs, let-7b and miR-223 were down regulated and miR-128a/b was upregulated in ALL as compared to AML. Cytogenetic risk groups in AML have also been shown to have differential microRNA expression signatures. Patients with cytogenetically normal AML (CN-AML) and favorable C/EBPα mutations had higher expression of miR-181 family members [135]. A specific microRNA signature was identified for AML with specific translocations, inversions or mutational subtypes. Specifically AML patients with t(8; 21) and inv(16) had higher levels of microRNAs let-7b and let-7c [136]; and lower levels of miR-126/126* [137] in their leukemic cells as compared to other patients. AML patients with normal cytogenetics and mutation in nucleophosmin (NPM1) gene frequently had elevated expression of miR-21 and subsequent downregulation of its target protein PDCD4 (programmed cell death 4) [138].

These and other studies focused on profiling microRNAs in AML have highlighted the complexity of this disease with miRNAs as another level of patient classification on top of cytogenetics and molecular abnormalities. These regulators can, not only influence the

expression of genes in AML subtypes but have also been shown to influence clinical outcome.

1.3.2.4. Role of MicroRNAs in Anticancer Drug Resistance

Anticancer drug resistance is a complex phenomenon since it could be either due to the intrinsic nature of the cancer cells or it may be due to the acquired resistance to the various process that are commonly targeted by these anticancer agents like insensitivity to drug-induced apoptosis, increased DNA repair, decreased drug accumulation, and induction of drug-detoxifying mechanisms [139]. Deregulation of microRNA expression in various cancers has been known to affect the expression of target mRNAs and/or proteins affecting various cellular processes, which might affect the chemosensitivity of cancer cells to various anticancer drugs. In order to understand the role of microRNAs in anticancer drug resistance, NCI-60 panel of cancer cell lines were screened for the effects of three microRNAs, let-7i, miR-16 and miR-21 on the chemosensitivities of various anticancer agents [140]. It was found that microRNAs could have diverse effects on the chemosensitivity an anticancer agent in cancers. Specifically it was found that increasing the levels of miR-21 in A549 cell line lead to increased sensitivity for cytarabine, which was opposite to what was observed with other anticancer agents, where decreased expression of miR-21 resulted in increased sensitivity of these agents, in this as well as other studies [140-142]. However, another study showed that inhibiting the expression of endogenous miR-21 enhanced the sensitivity of leukemic cell line (HL-60) to cytarabine primarily by increasing apoptosis [143]. Expression of miR-21 was associated with activation of PI3K/Akt/mTOR pathway and inhibition of miR-21increased sensitivity and

apoptosis induction by gemcitabine in cholangiocarcinoma and pancreatic cancer [141, 144]. The expression of miR-32 was observed to be markedly induced after treatment with 1, 25-dihydroxyvitamin D3 (1,26D) and this microRNA was also found to downregulate the expression of proapoptotic factor, Bim. Inhibiting the expression of miR-32 lead to increased sensitivity of AML cells to cytarabine [145]. Thus, microRNAs could alter cellular responses to anticancer drugs by affecting the cellular apoptotic response or survival pathways. MicroRNAs could also affect the chemosensitivity of cancer cells by affecting the drug targets, drug transporters and drug metabolizing enzymes. Increased expression of microRNA miR-27a and miR-451 was observed in multidrug resistant ovarian and cervical cancer cells as compared to their parental cell lines. Inhibition of these microRNAs increased the sensitivity of these cell lines to doxorubicin [146, 147]. Expression of another efflux pump, breast cancer resistant protein (BCRP) was found to be repressed by miR-328 in breast cancer cell lines, which lead to increased sensitivity of these cell lines to mitoxantrone [148]. Recently, a study showed that transfection of miR-130b mimic lead to significant downregulation of mRNA expression of various cytochrome P450s (CYP450s) namely, CYP1A1, 1A2, 2A6, 2C19, 2C8, and 2C9, phase II metabolizing enzymes like glutathione S-transferase alpha 2 (GSTA2), N-acetyltransferases (NAT1 and NAT2) and transporter solute carrier family 22 (Organic Anion Transporter), Member 7 (SLC22A7) and nuclear receptors, constitutive androstane receptor (CAR) and farnesoid X receptor. Additionally it was also demonstrated that miR-130b negatively regulated the activity levels of various CYP450s [149]. Another study showed that miR-148a negatively regulates the protein levels of

transcription factor pregnane X receptor (PXR) and miR-148a-dependent decrease in PXR levels attenuated the CYP3A4 induction [150]. These studies are a few of the many evidences, which demonstrate the significance of microRNAs in regulation of drug metabolism by regulating the expression/levels of transporters and drug metabolizing enzymes.

MicroRNAs are also known to have influence the anticancer drug resistance due to the single nucleotide polymorphisms (SNPs) that can influence microRNA functions. A SNP near the miR-24 binding site in the 3'UTR region of the dihydrofolate reductase (DHFR) gene resulted in loss of miR-24 mediated regulation of DHFR. The resulting increase of DHFR mRNA half-life was found to be associated with methotrexate resistance [151]. Drug resistance might also be associated with loss of microRNA binding sites on the 3'UTR of mRNA due to deletions. The inhibition of ABCG2 gene by miR-519c was lost in drug resistant cell lines harboring shorter 3'UTRs due to the loss of microRNA target sites, leading to overexpression of ABCG2 [152].

1.3.3. Drug-induced Changes

Anti-leukemic drugs, mainly nucleoside analogs act intracellularly by incorporating into the growing DNA strand. Due to this reason the intracellular uptake, activation, inactivation or changes in the dNTP pools could have an effect on the development of cellular resistance to these nucleoside analogs [153]. Although the intracellular triphosphate levels of these nucleoside analogs correlate with patient response in AML and small increases in the active triphosphate did show increased AML cytotoxicity, prior attempts in AML patients to increase cytarabine dosing did not improve clinical

outcomes [154]. Thus, mechanisms of resistance to nucleoside analogs could potentially involve additional mechanisms. A study on acute lymphoblastic leukemia (ALL) patients, analyzed the gene expression signatures before and after treatment with methotrexate and mercaptopurine, in order to understand the genomics of cellular responses to cancer treatment. Genes involved in apoptosis, mismatch repair, cell cycle control and stress response were identified to be differentially expressed in response to the different treatment groups [155]. Understanding the changes in gene expression post chemotherapeutic treatment can help in understanding the tumor adaptive response, which might be contributing to development of drug resistance. However these studies are very challenging to perform due to unavailability of specimens from patients after initiation of chemotherapy. A few global gene expression studies during neoadjuvant chemotherapy in primary breast cancer patients revealed that there was a distinct clustering of gene patterns post treatment, in patients with good pathological response to treatment compared to poor responders [156, 157]. Another study in breast cancer patients evaluated the chemotherapy-induced gene expression changes *in vivo*, post doxorubicin and docetaxel treatment, to provide insights into the resistance mechanisms of each drugs [158]. This study showed that the genes involved in protein and macromolecule metabolism were upregulated whereas those involved in cell cycle and DNA/RNA metabolism were downregulated in response to these two drugs.

So far there has been gap in our understanding of in vivo or in vitro gene expression changes in cancer cells after treatment with nucleoside analogs specifically in AML. Understanding the acute treatment-induced changes in the nucleoside analog pathway

genes in leukemic cells would offer new insights into additional mechanisms contributing resistance to these chemotherapeutic agents. The transcriptional response to nucleoside analog chemotherapy by assessing gene expression changes after drug treatment would also help better understand the cellular responses to injury, including the molecular pathways that initiate or block apoptosis and help identify additional drug targets.

1.4. Research Study Objectives

Despite extensive knowledge regarding the role of microRNAs as regulators of various cellular processes in cancer (prognostic markers for AML) and influencing changes in chemosensitivity of anti-cancer agents in *in vitro* systems; there have been very few studies that have systematically evaluated the role of microRNAs as predictors of chemosensitivity to nucleoside analogs in AML patients.

Thus, the major aim of this thesis project was to identify the role of microRNAs as potential markers of resistance to nucleoside analogs in the treatment of AML. The main objectives of this thesis are to:

1. Identify the microRNAs associated with chemosensitivity of cytarabine and determine the translational utility of these microRNAs as biomarkers for predicting response to cytarabine in AML patients.
2. Assess the effect of microRNAs on the expression of nucleoside analog pharmacokinetic and pharmacodynamic pathway genes and in turn assessing their potential impact on variability in response to nucleoside analogs.
3. Study the effect of *in vivo* cytarabine-induced changes in gene expression and to investigate the impact of these gene expression changes on clinical outcomes in AML patients.
4. Identify the microRNAs as predictors of chemosensitivity of a second generation nucleoside analog clofarabine in AML cell lines representing different risk groups.

CHAPTER II

MicroRNA-mRNA Pairs Predictive of Outcome in AML: From *in vitro* Cell-based Studies to AML Patients

2.1. Abstract

Cytarabine is the primary chemotherapeutic agent used for treatment of AML. Disease relapse after initial remission remains one of the most pressing therapeutic challenges in the treatment of AML. Relapsed disease is often resistant to cytarabine and subsequent salvage therapy is ineffective. Recent studies have shown that some miRNAs are associated with prognosis but have not yet explored the role of miRNAs in cellular response to cytarabine. We identified 20 miRNAs that associate with the *in vitro* cytarabine chemo-sensitivity or apoptotic response of eight AML cell lines. Out of the 20 miRNA, data on 18 miRNAs was available in AML patients from TCGA database. Our stepwise-integrated analyses (step 1- microRNA-target mRNA that were significantly correlated in AML patients; step -2 mRNAs from step 1 with significant association with OS) identified 23 unique miRNA-mRNA pairs predictive of OS in AML patients. As expected HOX genes (HOXA9, HOXB7 and HOXA10) were identified to be regulated by miRs as well as predictive of worse OS. Additionally, miR107-Myb, miR-378-granzyme B involved in granzyme signaling and miR10a-MAP4K4 were identified to be predictive of outcome through integrated analysis. Although additional functional validations to establish clinical/pharmacologic importance of microRNA-mRNA pairs are needed, our results from RNA EMSA confirmed binding of miR-10a, miR-378 and miR-107 with their target genes GALNT1, GZMB and MYB respectively. Integration of pathogenic and pharmacologically significant microRNAs and microRNA-mRNA relationships identified in our study opens up opportunities for development of targeted/microRNA-directed therapies.

2.2. Introduction

Acute myeloid leukemia (AML) is a hematological malignancy characterized by the presence of immature abnormal myeloid cells in bone marrow. It is a heterogeneous disease with various subtypes classified based on the morphology, immunophenotype and cytogenetics that are associated with outcome [1]. In spite of advances in recent years, 5-year overall survival is roughly 60% for children and ~25% for adults (Cancer Facts and Figures, American Cancer Society). Cytarabine (1-β-arabinofuranosylcytosine, ara-C), a nucleoside analog, is the most widely used chemotherapeutic agent used in combination with an anthracycline for treatment of AML. Cytarabine requires intracellular activation to form an active triphosphate metabolite that triggers apoptosis by inhibiting DNA synthesis. Although chemotherapeutic regimens including cytarabine induce complete response in 65 to 80% of AML patients, the majority of these patients suffer from disease relapse within 2 years of diagnosis. [2]. This can be partly attributed to the development of resistance of leukemic cells to cytarabine-based chemotherapy regimens. [3, 4]. Several factors such as molecular and cytogenetic subtype, differential gene-expression profiles, and epigenetics can account for development of resistance in AML [5-10]. We have previously identified gene expression signatures in AML patients predicting beneficial and detrimental patterns associated with cytarabine-based response [11]. Others have identified gene-expression differences between cytarabine-sensitive and -resistant cell lines in order to understand the molecular mechanisms underlying cytarabine resistance [12, 13].

MicroRNAs (miRNAs, miRs) are non-coding RNAs of 22-25 nucleotides that regulate gene expression. MicroRNAs bind to the complimentary sequence of messenger RNAs (mRNAs). In many cases, this binding suppresses mRNA translation or promotes mRNA degradation, thereby reducing expression at the protein level [14]. It has been shown that miRNAs play an important role in various cancers by regulating genes involved in cell proliferation, differentiation, and apoptosis [15-18]. Likewise, several recent studies have identified various miRNAs that differentiate the disease subgroups, associate with disease development, and associate with clinical prognosis of AML [19-25]. However, these valuable contributions have not yet carefully examined the roles of miRNAs in the cellular response of AML to cytarabine. Therefore, in this study, we characterized the association of miRNA expression with apoptotic response to cytarabine to identify candidate miRNAs for further evaluation of their relationship with mRNAs and overall survival in The Cancer Genome Atlas (TCGA) cohort of 200 AML patients.

2.3. Materials and Methods

Figure 2.1 shows overall study design.

Cell Culture and Reagents: The AML cell lines HL-60, MV-4-11, Kasumi-1, THP-1, AML-193, and KG-1 were obtained from ATCC (Manassas, VA), while ME-1 and MOLM-16 cell lines were obtained from DSMZ (Braunschweig, Germany). All the cell lines were cultured in the media as recommended by the supplier and were maintained in a humidified incubator at 37°C with 5% CO_2. The cells were passaged every 2 to 3 days in order to maintain them in logarithmic growth phase. Cytarabine and the 3-(4, 5-

dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent were purchased from Sigma Aldrich (St. Louis, MO). Stock concentrations for cytarabine (5mg/ml) were prepared in sterile water and stored in -20°C in aliquots.

2.3.1. Cytotoxicity Assay: Cytarabine cytotoxicity was determined using the MTT assay. Briefly, AML cell lines were plated in 96-well plate at seeding density of 2.5×10^5 cells/ml and incubated at 37°C overnight. After 24h of recovery time, the cells were exposed to varying concentrations (200, 100, 50, 5, 0.5, 0.01 and 0 μM) of cytarabine. Cell viability was determined 48h post cytarabine treatment by incubation with MTT reagent followed by measuring the absorbance at 570nm using Synergy plate reader (BioTek, USA). The percent cell survival at each concentration was calculated using the Gen5™ Software version 1.11 (Winooski, VT). The area under the survival curve (AUC) was calculated by the trapezoidal method using the GraphPad Prism software version 6 (La Jolla, California).

2.3.2. Apoptosis Assay: The apoptotic activity of AML cell lines following treatment (48 h) with varying concentration of cytarabine (as indicated above) was determined using the Caspase-Glo® 3/7 assay as per manufacturer's instructions (Promega, USA). 48h post cytarabine treatment, luminescence was read using Synergy plate reader (BioTek, USA). The luminescence produced is directly proportional to the caspase activity. The caspase activity at each concentration was normalized to the control and the area under the relative caspase activity curve (AUC) was calculated by the trapezoidal method using the GraphPad Prism software version 6 (La Jolla, California).

2.3.3. MicroRNA Expression Analysis: For determination of microRNA expression, total RNA was isolated using mirVana™ miRNA Isolation kit (Life Technologies, USA) as per the manufacturer's protocol. The RNA quality and concentration was measured using NanoDrop 2000 UV-Vis spectrophotometer (ThermoScientific, USA). A total of 100 ng of purified total RNA was used for nCounter miRNA sample preparation reactions according to manufacturer's instructions and was assayed for determination of 800 human microRNA expressions using the nCounter Human v2 miRNA Expression Assay kit (Nanostring Technologies, USA). Preparation of small RNA samples involved multiplexed ligation of specific tags (miRtags) to the target microRNAs that provide unique identification for each microRNA species. After ligation, the detection was done by hybridization to microRNA: tag specific nCounter capture and barcoded reporter probes. Data collection was carried out using the nCounter Digital Analyzer (Nanostring Technologies, USA) at The University of Minnesota Genomics Center, following manufacturer's instructions to count individual fluorescent barcodes and quantify the target microRNA molecules present in each sample. MicroRNA expression data normalization was performed using the nSolver™ Analysis Software (Nanostring Technologies) according to the manufacturer's instructions. In particular, the data was normalized using the expression of the top 100 codesets. Further, to account for the background correction, mean of negative controls plus two standard deviation (SD) method was used. In order to avoid using the microRNAs with a very low expression, we further filtered out the microRNAs with expression counts <30 (2 times the mean ± 2 SD of negative control value), in order to account for the background noise. Total 412

microRNAs with expression counts >30 were evaluated for differential expression between sensitive and resistant cell lines and for their correlation with cytarabine chemosensitivity.

2.3.4. The Cancer Genome Atlas (TCGA) Data: The clinical outcome data, mRNA expression and microRNA expression data for AML patients was extracted from The Cancer Genome Atlas (TCGA) Data Portal (cancergenome.nih.gov/) [26]. Out of the 200 AML patients in TCGA database, 197 patients had gene expression profiling data available and 187 patients had microRNA expression data available. 186 patients had both gene expression and microRNA expression data available. Out of the 186 AML patients, 13 patients lacked valid survival information and 2 patients lacked cytogenetically defined risk information. Thus, data for a total of 186 patients were used to evaluate miRNA-mRNA associations, 173 patients used to evaluate mRNA-OS associations and miRNA-OS associations (171 for stratified analyses).

2.3.5. Statistical Analysis: For each miRNA, Spearman's rank-based correlation was used to measure the association with cytarabine treatment response or apoptosis in the eight cell lines. For each miRNA-mRNA pair with predicted binding sites defined by miRBase (www.mirbase.org; release 21), Spearman's rank-based correlation was used to evaluate the association of miRNA expression with mRNA expression on the TCGA AML cohort. The p-value for the Spearman statistic was determined by 10,000 permutations. For each miRNA or mRNA, Cox regression (or Jung's statistic) was used to evaluate the association of expression with overall survival (OS). FDR was estimated by Pounds and Cheng's robust FDR method [27].

Electrophoretic Mobility Shift Assays: The functional validation for binding efficiencies between selected microRNAs and mRNAs was performed using the electrophoretic mobility shift assays (EMSAs) as described previously [28]. The binding free energy between the respective mRNA and microRNA pair (demonstrating inverse relationship) was predicted using the RNAhybrid software. The microRNA oligonucleotides were labeled with cy5™ dye on their 5' ends. The 2' O-methyl-modified mRNA oligonucleotides were labeled with IRDye®800 (LI-COR Biosciences, USA) dye on their 5' ends. The labeled oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, USA). RNA EMSA experiment was performed using the LightShift Chemiluminescent RNA EMSA Kit (ThermoScientific, USA) according to the manufacturer's protocol. The mRNA oligonucleotide was heated for 10 minutes at 80°C and then placed on ice in order to relax the secondary structures. In each 20µl binding reaction, 200nM microRNA oligonucleotide and /or mRNA oligonucleotide were mixed with RNA EMSA binding buffer and incubated at 25°C for 25 minutes. The reaction mixtures were separated on a 15% polyacrylamide gel by electrophoresis at 4°C. The binding reactions were electrophoretically transferred onto nylon membrane and the resulting mobility shifts were imaged using and Odyssey CLx Infrared System (LI-COR Biosciences, USA).

2.4. Results

2.4.1. Cytarabine chemosensitivity of AML cell lines: The AML cell lines showed considerable variability in cytarabine sensitivity as measured by cytotoxicity AUC and

apoptosis AUC in the MTT assay (Table 2.1; Supplementary Fig. 2.1A-1H). Based on the cytotoxicity AUC, HL-60, MV-4-11, KG-1 and ME-1 were classified as sensitive (cytarabine AUC < 12000), while MOLM-16, AML-193, Kasumi-1 and THP-1 were classified as resistant cell lines (cytarabine AUC > 12000).

2.4.2. MicroRNAs associated with cytarabine chemo-sensitivity in AML cell lines: Of the 800 human microRNAs quantitated using the nCounter Nanostring platform we excluded 388 miRNAs due to very low expression and 412 miRNA were analyzed further. Twenty miRNAs were associated with cellular viability (9 miRs) or caspase activation (11 miRs) in AML cell lines post treatment with cytarabine (Table 2.2). Expression of miR-25-3p, miR-148b-3p, miR-107, miR-374-5p, miR-425-5p were positively associated with AUC for cell survival post-cytarabine treatment and miR-16-5p, miR-24-3p, miR-196a-5p and miR-155-5p were negatively associated with AUC for cell survival post-cytarabine treatment ($p< 0.05$). (Selected miRNAs are shown in supplementary Fig. 2.2) Expression levels of miR-10a-5p, miR-29a/b-3p, miR-30e-5p, miR-33a-5p, miR-378a/g were positively and expression levels miR-197-3p, miR-27b-3p, miR-324-5p and miR-421 were negatively associated with AUC for caspase3/7 activation (apoptosis) post cytarabine treatment (Table 2.2, $p<0.05$).

2.4.3. Pairs of significantly correlated mRNAs and miRNAs that associate with overall survival of AML Patients: Of 20 miRNAs identified above, data on 18 were available in AML patients from TCGA database and were tested for associations with risk group and outcome. As shown in Supplementary Table 2.1, seven of these miRNAs (miR-10a, miR-16, miR-196a, miR-197, miR-421, miR-155 and miR24) demonstrated

significant difference in expression levels among the three risk groups. In risk stratified analysis miR107, miR-155, miR-196a, miR-25 and miR29b were associated with worse OS whereas miR-25 was predictive of better OS in AML patients at $p<0.05$.

Using miRBase (www.mirbase.org; release 21), we determined that 5006 probe sets representing 2830 gene with binding sites for these 18 miRNAs. These 5006 mRNAs and 18 miRNAs belong to 7132 distinct miRNA-mRNA pairs. Using the analysis strategy outlined in Fig. 1, we found that 23 of the 7132 miRNA-mRNA pair's satisfied criteria listed below (Table 2.3):

(a) significant association between miRNA and target mRNA ($p < 0.05$; 1532 pairs) and

(b) mRNA expression associated significantly with overall survival in an unstratified Cox regression model ($p < 0.001$; FDR < 0.05).

These 23 pairs included 16 unique genes and 10 unique miRNAs (Table 2.3 -some mRNAs and some miRNAs belonged to multiple pairs). A positive correlation of mRNA and miRNA was observed for 10 of these pairs and a negative correlation was observed for the other 13 pairs.

Among mRNA-OS associations COL3A1, GALNT1, GALNT7, LTK, MAP4K4, MYB, PAPOLG, RPL35A, TMEM87A and WDR48 were associated with better OS and HOX family genes (HOXA9, HOXA10, HOXB7), GZMB, SE1L3 and an oncogene PIM1 were associated with inferior outcome (Table 2.3). Since nine of these genes demonstrated significant association with risk groups we also performed risk-stratified analysis and all but 3 genes HOXA9, HOXA10 and LTK were significantly associated

with OS in risk stratified analysis, indicating that for these genes the observed association with OS might be driven by risk group characteristics. Fig. 2.2 shows the representative correlation plots as well as overall curves for miR10a-GALNT1, miR10a-MAP4K4 and miR16-Pim1, miR378-GZMB and miR107-MYB.

We further utilized Ingenuity Pathway analysis tool to map the 16 unique genes identified in integrative analysis and as shown in Fig. 2.3 these genes were associated with cell proliferation, apoptosis, RNA expression, and quantity of hematopoietic progenitor cells, hematological cancer and myeloid leukemia.

2.4.4. Functional validation using RNA Electrophoretic mobility shift assays (RNA EMSA): To further validate the miRNA-mRNA pairs discovered as above, we performed RNA EMSAs. We first selected miRNA-mRNA pairs from Table 2.3 that demonstrated significant inverse relationships. Binding free energy for these miRNA-mRNA pairs was calculated using RNAhybrid software The 3'UTR sequence of mRNA was obtained from the UCSC Genome browser and microRNA sequence was obtained from miRBase software. Of miRNA-mRNAs demonstrating minimum free energy of binding <-24 kcal/mol miR107-MYB; miR378a-GZMB and miR10a-GALNT1 were validated by RNA EMSAs. EMSA results confirmed binding for miR-107-MYB, miR-10-GALNT1 and miR-378- GZMB and are shown in Fig. 2.4A, 2.4B and 2.4C respectively. As shown in lane 3 in Fig. 2.4A, 2.4B and 2.4C, the EMSA results for miR-107, miR-10a and miR-378 demonstrate binding of these miRNAs with their respective mRNA target sequences confirming the thermodynamic stability of these complexes predicted in the in silico analysis. In addition, we observed competition of binding in mRNA-microRNA

complexes after adding excess unlabeled specific miRNA probe (Fig. 2.4A-C, lane 4), excess unlabeled mRNA probe (Fig. 2.4A-C lane 5) but not by adding excess unlabeled non-specific probe (Fig. 2.4A-C, lane 6).

2.5. Discussion

AML is a heterogeneous disease with dismal outcome. Additional complexity is added by very heterogeneous nature of AML with cytogenetic abnormalities used for risk classification in AML patients. Although cytarabine has been the backbone of AML chemotherapy for more than 50 years, there are still gaps in our understanding of the molecular mechanisms contributing to development of drug resistance in AML. Although advances in supportive care have improved, the treatment strategies have not changed much with cytarabine being still the main player. Thus, understanding the molecular mechanisms underlying cytarabine resistance will be of great interest in developing predictive models of outcome as well as for developing novel therapeutic strategies Recent research has shown the significant role of microRNAs in normal hematopoiesis [29-31] as well as microRNA deregulation in AML. MicroRNAs (miR-125, miR-146, miR-142, miR-155, miR-29, miR- 181, let-7a etc.) of potential prognostic significance have been identified [32], however the role of microRNAs in development of resistance to cytarabine and thus inferior clinical outcome has not been investigated in detail. In this study we report results of our genome-wide evaluation to identify microRNAs associated with cytarabine sensitivity in 8 AML cell lines as well as their impact on clinical outcome in AML patients (Fig. 2.1 outlines overall study design and results).

We screened expression levels of 800 human microRNAs in eight AML cell lines and after filtering out microRNAs with very low expression, evaluated 412 microRNAs for association with cytarabine chemo-sensitivity, measured as cell viability and apoptosis induction following cytarabine treatment. Twenty unique microRNAs were predictive of cytarabine chemo-sensitivity in AML cell lines, and 18 of these were further evaluated in AML patients from TCGA database. Seven of these were differentially expressed among AML risk groups (favorable, intermediate and poor; $p<0.05$) and after risk stratification 5 miRNAs (miR-107, miR-155, miR-25, miR-29b and miR-196a) were associated with OS (Supplementary Table 2.1). All but miR-25 were associated with worse OS in AML patients.

Among the miRNAs associated with OS, miR-155 is located in a non-coding RNA transcript cluster called B-cell integration cluster (BIC), which has been shown to cooperate with c-Myc [33, 34]. miR-155 is considered as an oncomiR with implications in pathogenesis of AML [16, 31]; it has been associated with SHIP1 (negatively regulator of PI3K/AKT pathway) and CEBP-β [35, 36]. Our results shows that miR-155 is associated with cytarabine sensitivity which is not in consensus with its association with inferior outcome, thereby indicating that miR-155 (which is also differentially expressed among risk groups) might have significant impact on disease pathogenesis but might not be impacting drug response. miR-29 family members are regulators of myeloid differentiation and have been shown to be deregulated in AML [37-39]. MiR29a/29b have been associated with expression levels of oncogenes MCL1, CDK6, IGFR and JAK2 [38, 40] as well as have been shown to target DNA modifying genes DNMTs and

TET2 [41, 42].

In step-wise integrated analysis we identified 23 miRNA –mRNA pairs predictive of survival in AML patients and these pairs were mapped to 16 unique mRNAs.

We further validated miR107-MYB, miR-10a-GALNT1 and miR378-GZMB miRNA-mRNA pairs using electrophoretic mobility shift assays (Fig. 2.4), which confirmed the binding of these oligos as supported by *in silico* analysis indicating miRNAs in regulating gene expression of these target genes by binding to specific seed sequences.

As expected HOX genes (HOXA9, HOXA10 and HOXB7) were predictive of worse outcome. miR-196a-1 which was associated cytarabine in vitro sensitivity in AML cell lines was positively correlated in expression with HOXA9 and HOXB7 as well as with AML risk groups. miR-196a-1 gene co-localizes with HOXB gene cluster between HOXB8 and HOXB13, positive correlation observed in AML patients between HOXB7 and mir-196a might be due to transcriptional co-regulation. miR-196a has been previously shown to be positively correlated with several HOX family members including HOXB7 and HOXA9 [43]. In addition to miR-196a, we observed significantly negative correlation between miR-16-HOXA10, miR-421-HOXA9 and miR-30e-HOXB7.

Among other miR target genes that were associated with worse OS were PIM-1 (pim1 oncogene), GZMB (granzyme B), and SEL1L3 (Sel-1 Suppressor Of Lin-12-Like 3). Pim1 is a serine/threonine protein kinase that has role in cell survival and cell proliferation. HoxA9 is transcriptional activator of Pim-1, which is further involved in regulation of MYC transcriptional activity, regulation of cell cycle progression and

phosphorylation and inhibition of proapoptotic proteins (BAD, MAP3K5, FOXO3) thereby by contributing to its oncogenic activity. Pim-1 is also involved in inactivating MAP3K5 by phosphorylation thereby inhibiting MAP3K5-mediated phosphorylation of JNK and JNK/p38MAPK subsequently reducing caspase-3 activation and cell apoptosis. Pim-1 seems like a potential target for drug discovery, in fact in pediatric preclinical models, Pim1 inhibitor SGI-1776 has been shown to induce complete response to subcutaneous MV4:11 leukemia[44] as well as inhibit proliferation in other malignancies such as CLL, B cell lymphoma, multiple myeloma etc. [45-47].

GZMB was negatively regulated by miR-378 and higher expression was associated with worse OS in AML. GZMB is a key player in Granzyme signaling pathway, which is a lymphocyte granular serine protease that cleaves its substrates at Asp residues. GZMB is expressed in cytotoxic T lymphocytes (CTL) and NK cells and is primary mediator of apoptosis by CTL in cell-mediated immune response. GZMB seems to play critical role antibody –dependent cellular cytotoxicity [48].

Among miR target genes that were associated with good response were family members of Polypeptide N-Acetylgalactosaminyltranferases (GALNT1 and GALNT7), MAP4K4, TMEM87A and COL3A1. GALNT1 was correlated negatively in expression with miR-10a, miR-16-2 and positively with miR-30e, which also demonstrated positive correlation with GALNT7 expression. MAP4K4 belongs to serine/threonine protein kinase family and has been specifically implicated in activation of MAPK8/JNK pathway.

Both MYB and TMEM87 were inversely associated with miR-107, which was associated with cytarabine resistance in cell lines as well as worse overall survival in AML patients

(Tables 2.2 and 2.3). Oncogenic role of miR-107 in regulating tumor invasion and metastasis in gastric cancer by targeting DICER1 [49] and in colorectal cancer by targeting metastasis suppressors death-associated protein kinase (DAPK) and Krüppel-like factor 4 (KLF4) [50] has been proposed. In AML patients, we observed negative correlation of DICER with miR-107 ($p<0.01$) although DICER expression was not predictive of outcome. Although TMEM87A a transmembrane protein has not been well studied, MYB, a V-Myb Avian Myeloblastosis Viral Oncogene Homolog has been implicated in leukemogenesis. Myb is reported to be overexpressed in AML and results from recent studies shows its potential role in interplay between C/EBP activity for transcriptional regulation of FLT3 expression [51]. Recent report in luminal breast cancer demonstrated for the first time the potential tumor suppressor role of c-Myb gene, [52], which is in concordance with TCGA results with Myb expression associated with better OS thereby warranting further investigation of Myb gene on its impact on treatment outcome in AML.

In summary, although several studies have established prognostic significance of microRNAs (miR-155, miR-29, miR-16, miR 223 etc.) in AML, role of microRNAs in cytarabine chemosensitivity and development of resistance as a contributor of inferior outcome has not been well studied. In this report we performed genome-wide microRNA profiling of 8 AML cell lines and identified 20 miRNAs predictive of differential AML in vitro chemo-sensitivity (by measuring both cytarabine induced cell death and apoptosis). These were further investigated in AML patients using data from TCGA database, and in an integrated three way analyses of miRNA- target mRNA pairs with significant

association and OS we identified 23 microRNA-mRNA-OS pairs of therapeutic importance in AML patients. Although additional functional validation studies to establish clinical/ pharmacological importance of microRNA-mRNA pairs are needed, our preliminary data on RNA EMSAs confirmed binding of miR-107-MYB, miR10a-GALNT1 and miR-378-GZMB. Integration of pathogenic and pharmacologically significant microRNAs and microRNA-mRNA relationships opens up opportunities for development of targeted/microRNA-directed therapies.

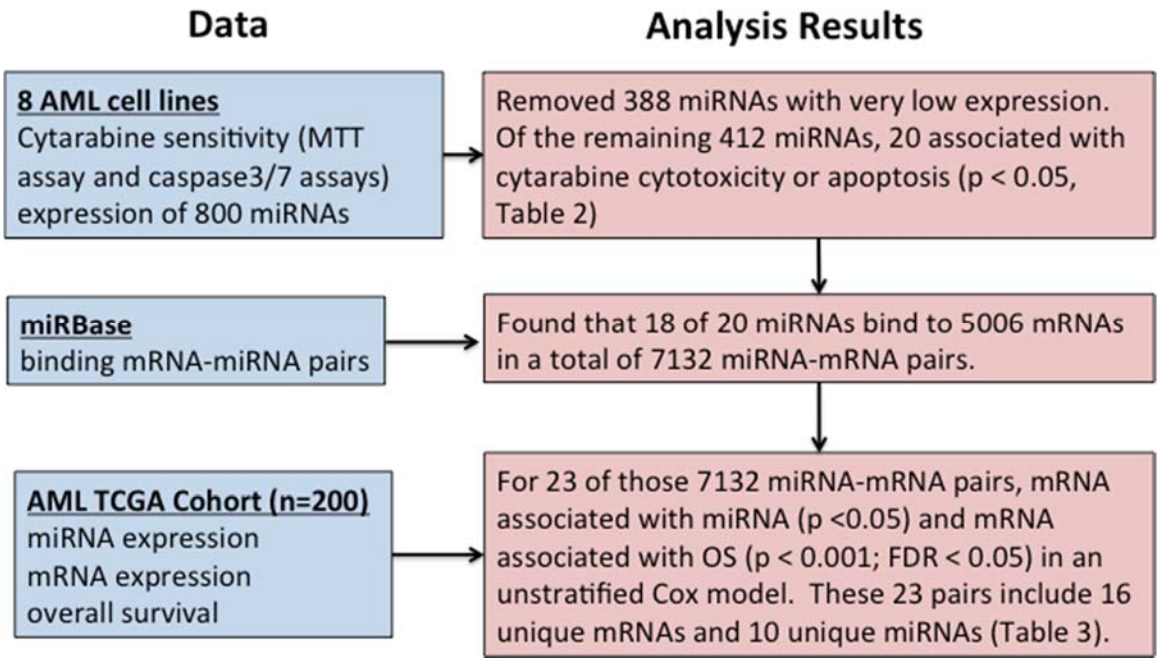
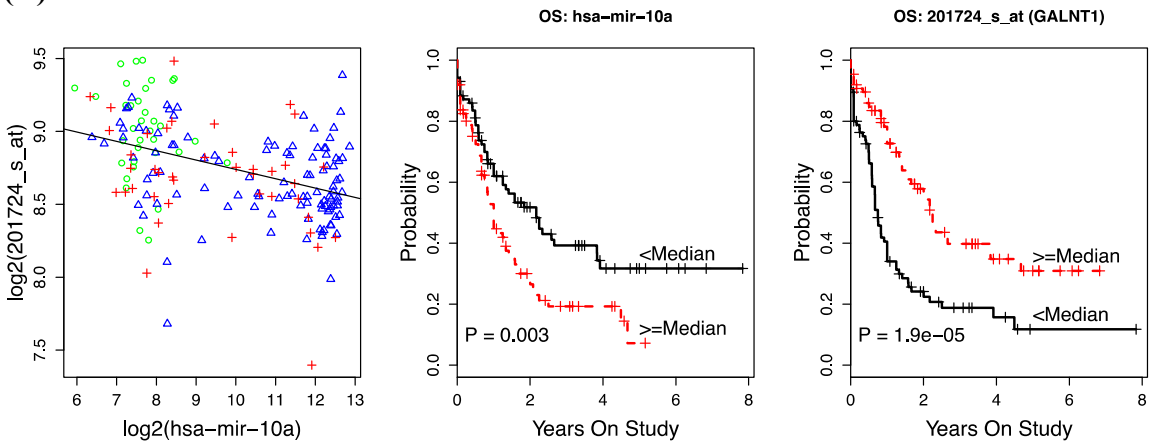
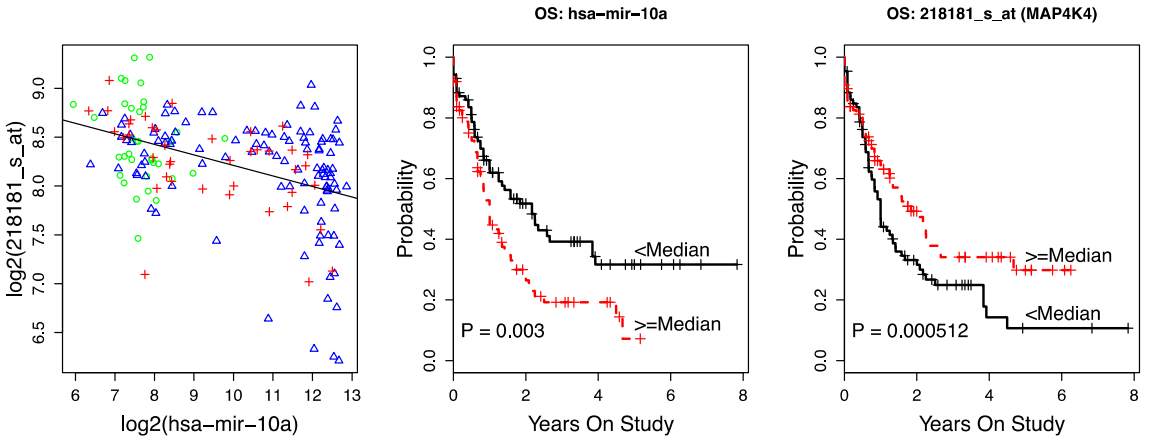


Figure 2.1: Overall schema of the proposed study. Study Design for identification of microRNAs influencing cytarabine chemo-sensitivity and survival in AML patients.

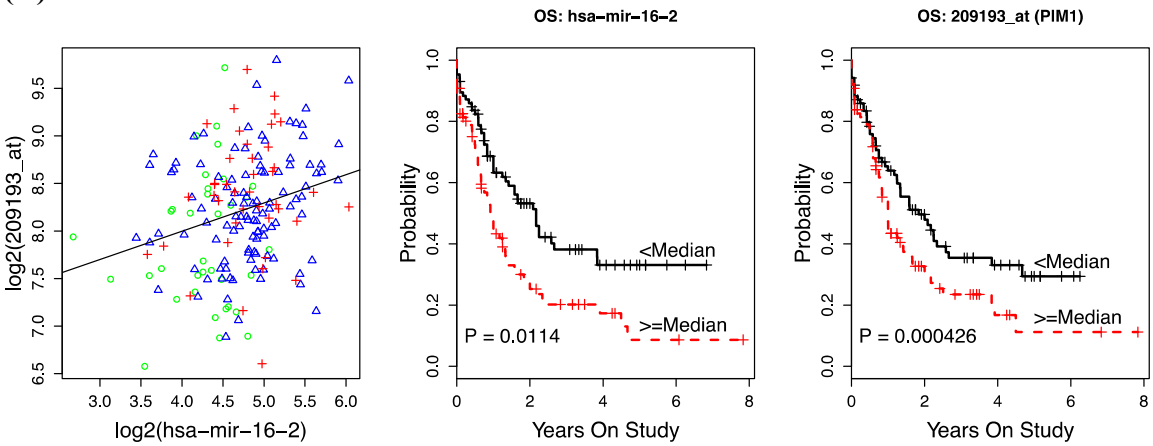
(A) miR-10a and GALNT1



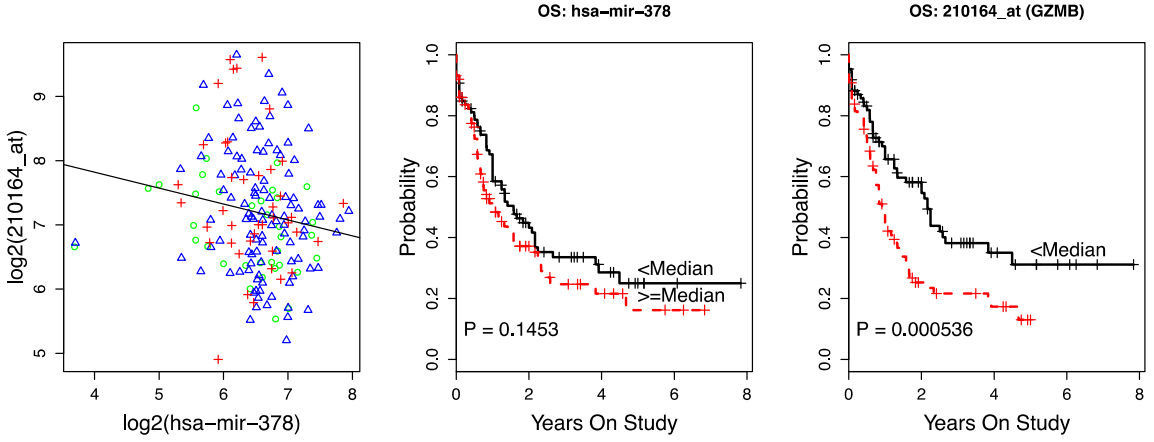
(B) miR-10a and MAP4K4



(C) miR-16 and PIM-1



(D) miR-378 and GZMB



(E) miR-107 and MYB

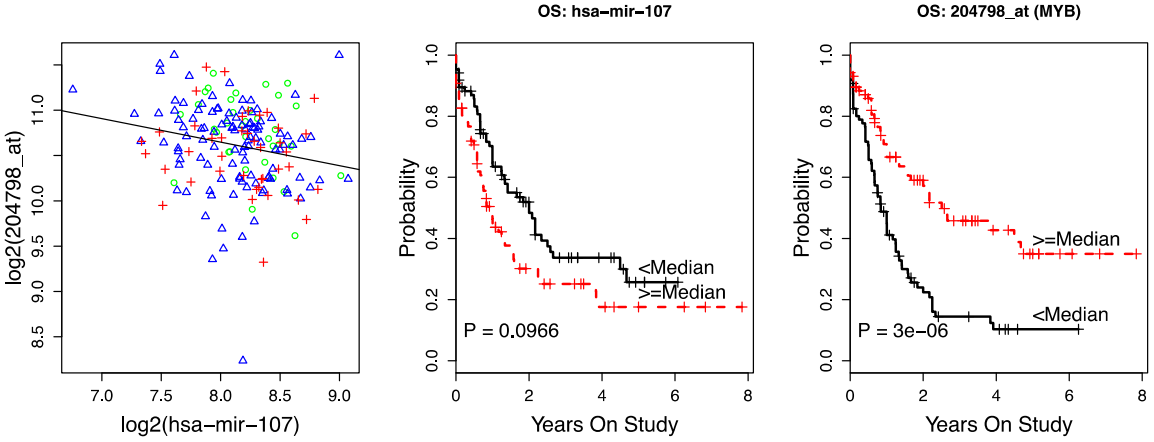


Figure 2.2: Representative plots showing correlation between miRNA- target mRNA and survival curves of miRNA and mRNA expression with OS in AML patients from TCGA database. A) Correlation plot of miR-10a-GALNT1 mRNA levels and Kaplan Meier survival curves of miR10a and GALNT1 expression with OS. B) Correlation plot for miR-10a-MAP4K4 mRNA levels and Kaplan Meier survival curves of miR-10a and MAP4K4 expression with OS. C) Correlation plot of miR-16 and Pim1 mRNA levels and Kaplan Meier survival curves of miR-16 and PIM1 expression with OS D) Correlation plot of miR-378 and GZMB mRNA levels and Kaplan Meier survival

curves of miR-378 and GZMB expression with OS. E) Correlation plot of miR-107 and MYB mRNA levels and Kaplan Meier survival curves of miR-107 and MYB expression with OS.

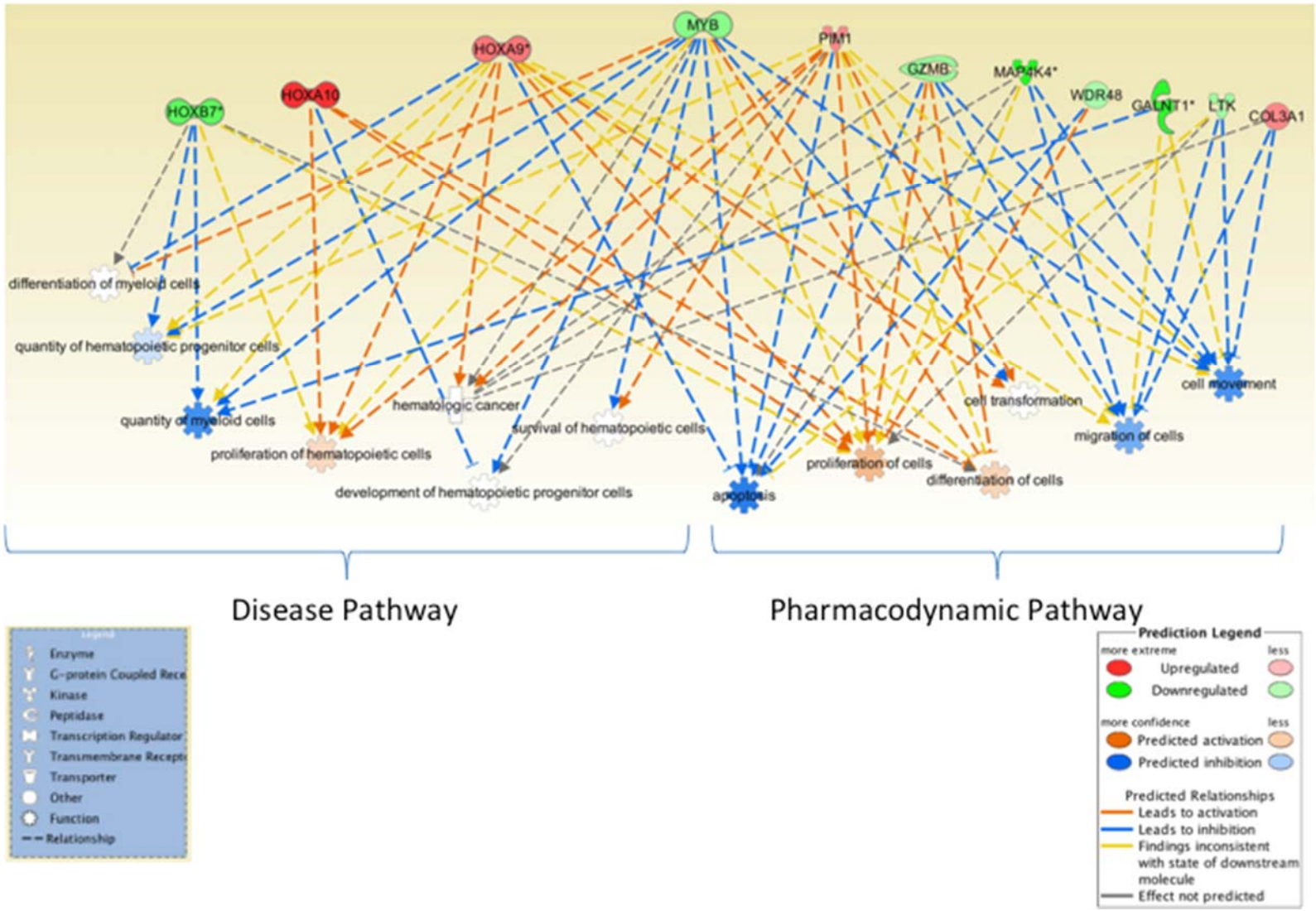

MYB
HOXA9*
PIM1
GZMB
MAP4K4*
WDR48
GALNT1*
LTK
COL3A1
HOXB7*
HOXA10
differentiation of myeloid cells
quantity of hematopoietic progenitor cells
quantity of myeloid cells
hematologic cancer
survival of hematopoietic cells
proliferation of hematopoietic cells
development of hematopoietic progenitor cells
apoptosis
proliferation of cells
cell transformation
differentiation of cells
migration of cells
cell movement
Disease Pathway
Pharmacodynamic Pathway
Enzyme
G-protein Coupled Rece
Kinase
Peptidase
Transcription Regulator
Transmembrane Recepto
Transporter
Other
Function
Relationship
Prediction Legend
more extreme
less
Upregulated
Downregulated
more confidence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted

Figure 2.3. Network analysis. Ingenuity Pathway analysis tool was utilized to evaluate the genes identified in miRNA-mRNA-OS analysis. Several genes mapped to biological processes of relevance to hematological malignancies, myeloid leukemia as well as to cell proliferation and apoptosis, RNA expression. Genes associated with better OS are in green and ones with worse OS are in red.

(A)

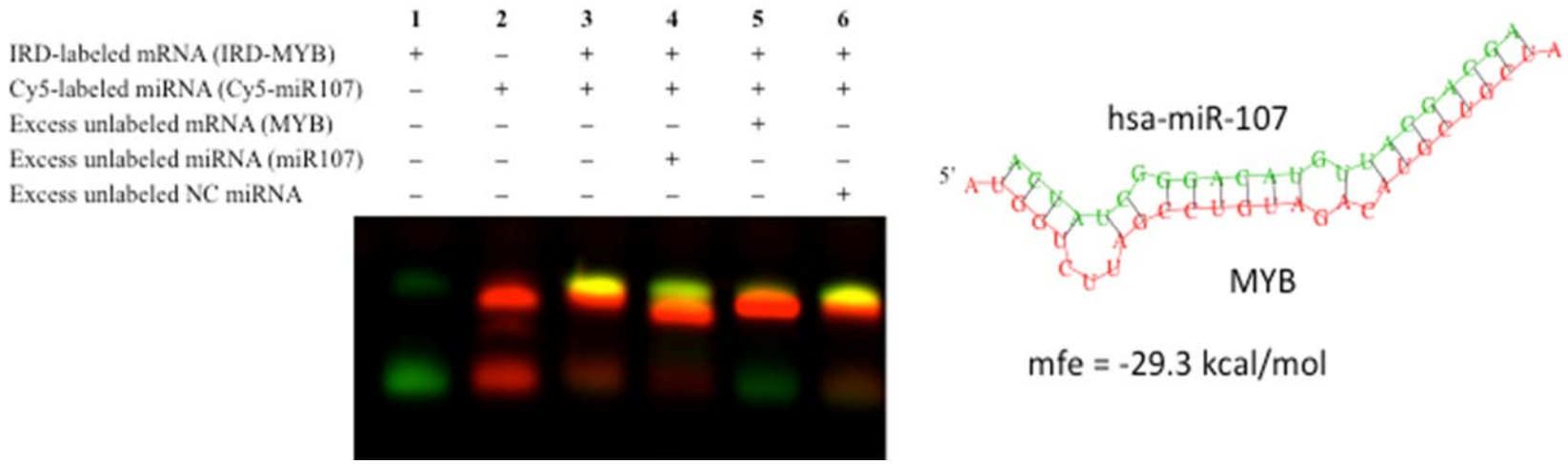


Figure 2.4. (A) Validation of binding interaction between miRNA-mRNA by RNA Electrophoretic Mobility Shift Assays: Validation of binding interaction between MYB mRNA and hsa-miR-107 by RNA EMSAs. RNA EMSA with cy5-labeled hsa-miR-107 oligonucleotide and 2'-O-methyl modified and IRD-800 labeled MYB mRNA oligonucleotide. Lanes 1 and 2 show the mobility of the labeled mRNA or microRNA oligonucleotide. Lane 3 shows the mobility of the labeled hsa-miR-107 oligonucleotide with MYB mRNA oligonucleotide. Lanes 4 and 6 show the mobility of labeled MYB mRNA oligonucleotide in presence of unlabeled excess specific competitor (hsa-miR-107) and excess unlabeled non-specific competitor (NC)

(B)

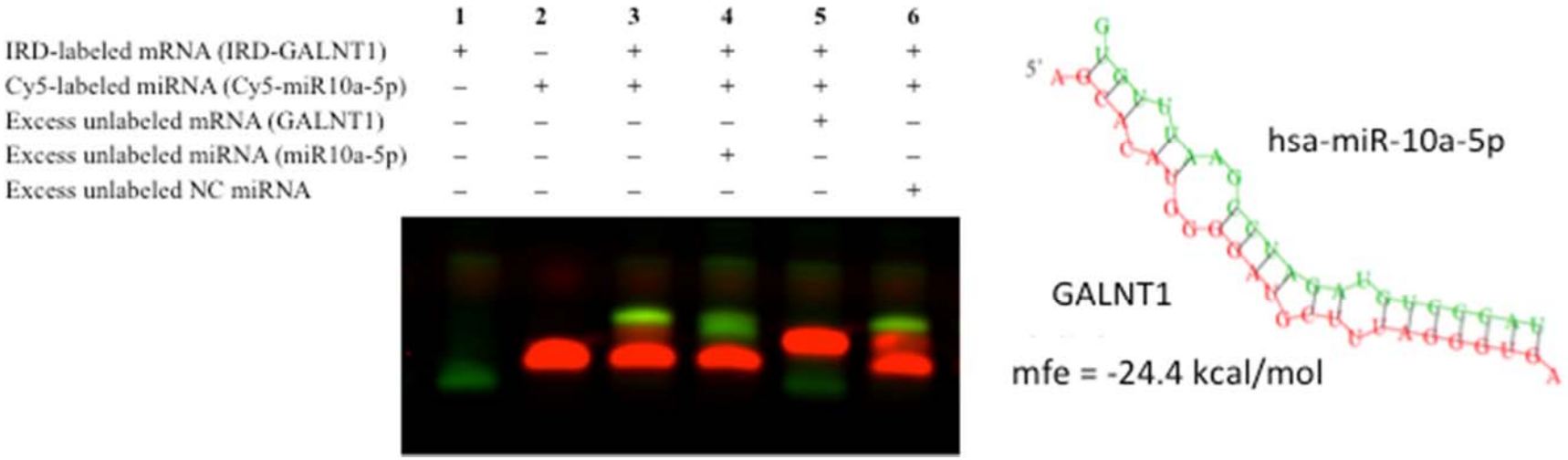


Figure 2.4. (B) Validation of binding interaction between miRNA-mRNA by RNA Electrophoretic Mobility Shift Assays: Validation of binding interaction between GALNT1 mRNA and hsa-miR-10a-5p by RNA EMSAs. RNA EMSA with cy5-labeled hsa-miR-10a-5p oligonucleotide and 2'-O-methyl modified and IRD-800 labeled GALNT1 mRNA oligonucleotide. Lanes 1 and 2 show the mobility of the labeled mRNA or microRNA oligonucleotide. Lane 3 shows the mobility of the labeled hsa-miR-10a-5p oligonucleotide with GALNT1 mRNA oligonucleotide. Lanes 4 and 6 show the mobility of labeled GALNT1 mRNA oligonucleotide in presence of unlabeled excess specific competitor (hsa-miR-10a-5p) and excess unlabeled non-specific competitor (NC)

(C)

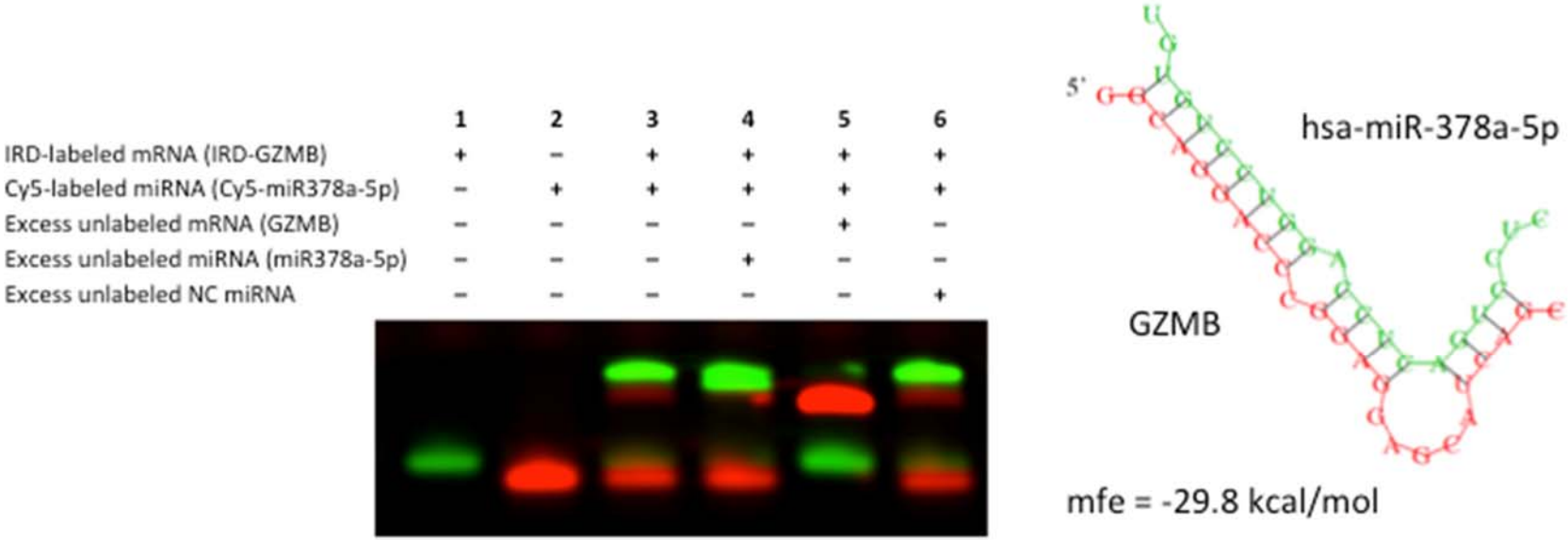
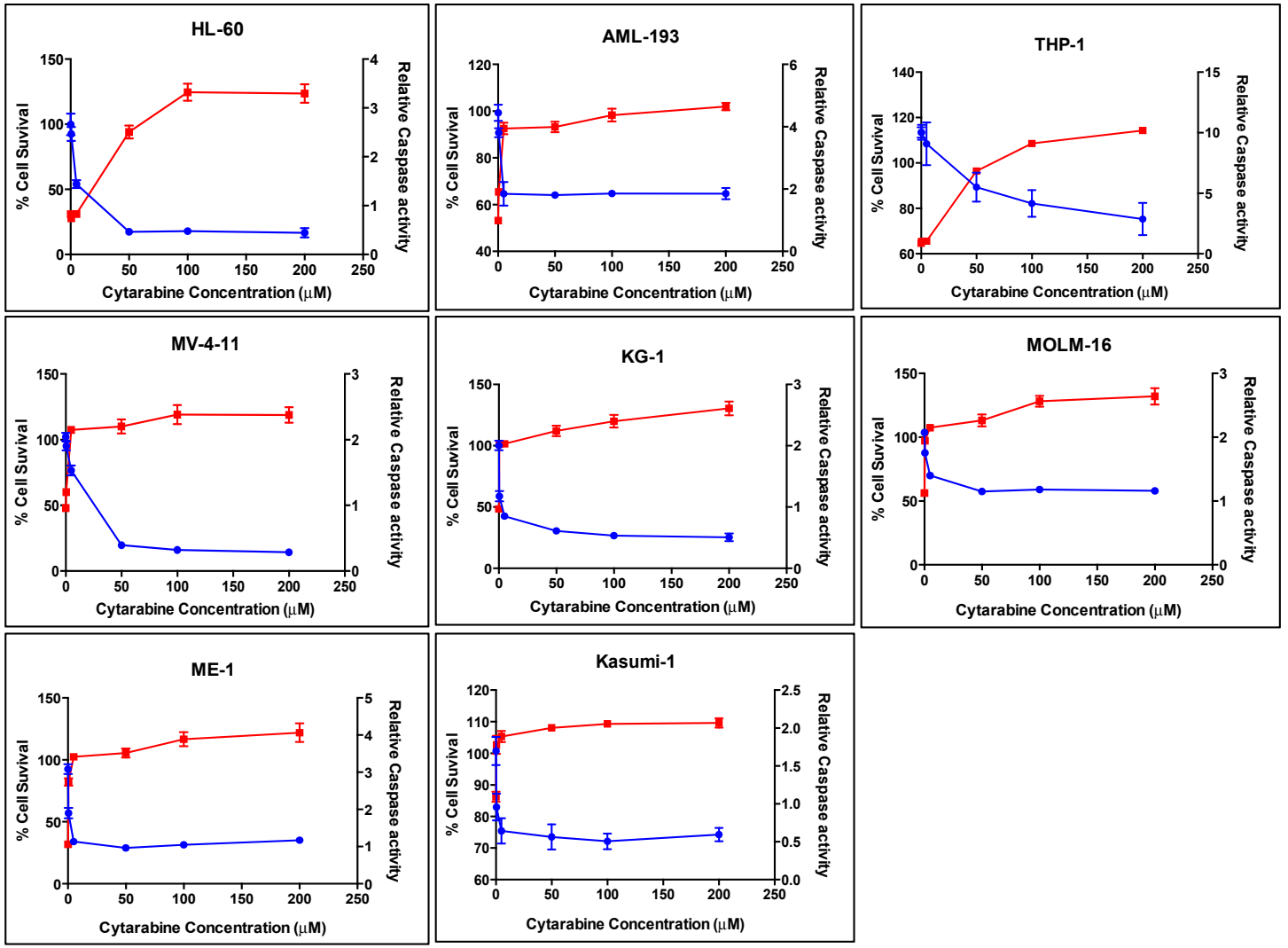
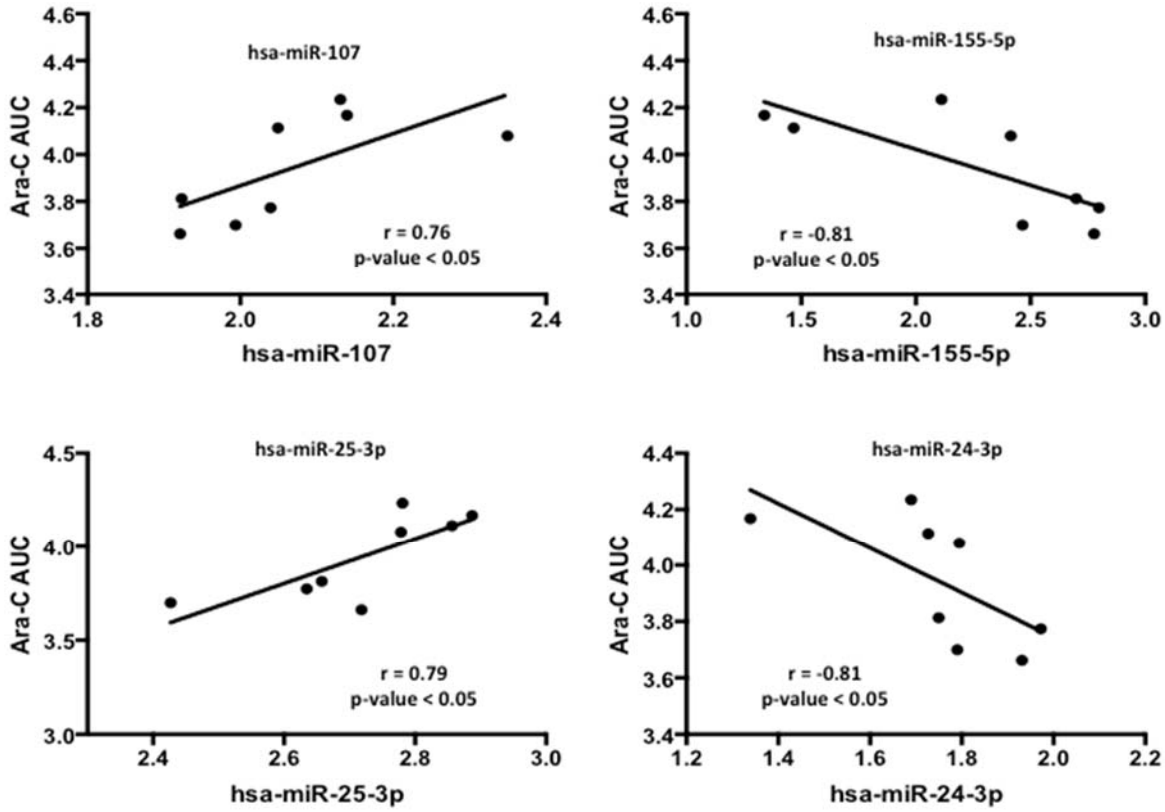


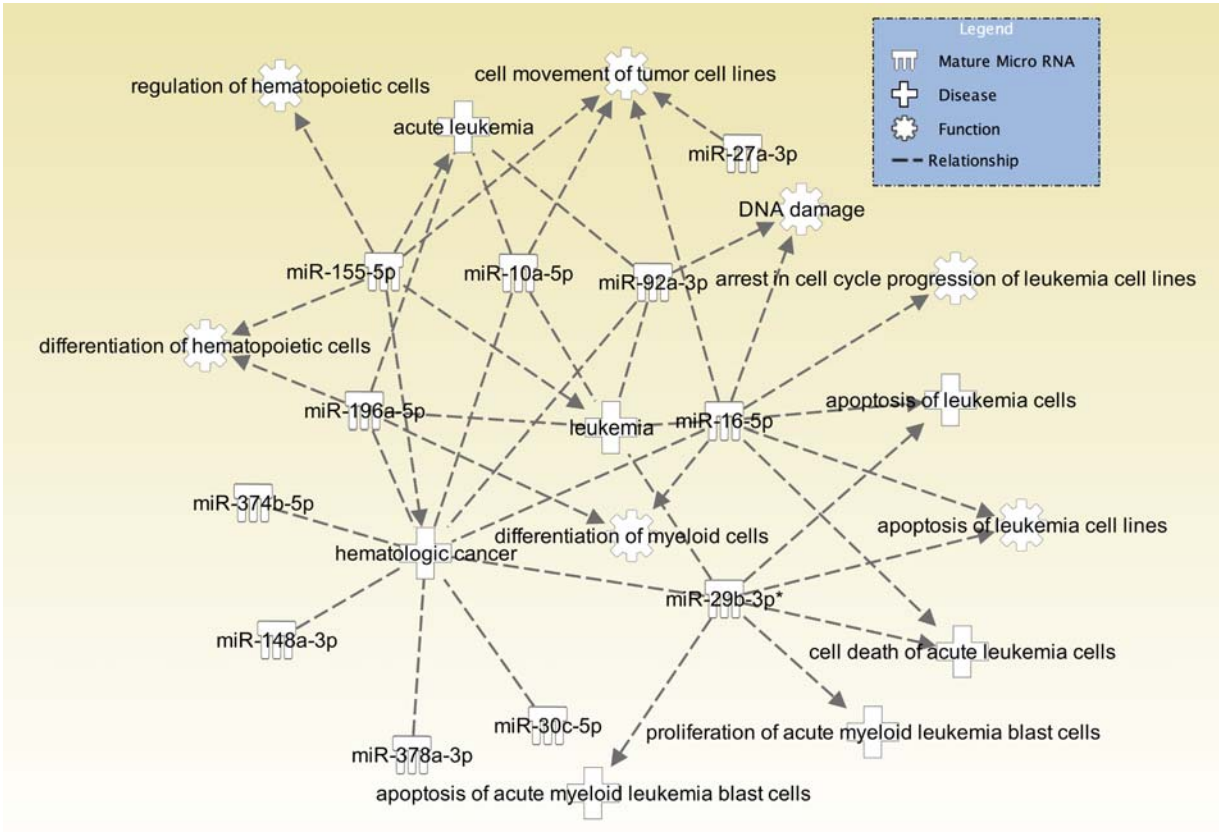
Figure 2.4. (C) Validation of binding interaction between miRNA-mRNA by RNA Electrophoretic Mobility Shift Assays: Validation of binding interaction between GZMB mRNA and hsa-miR-378a-5p by RNA EMSAs. RNA EMSA with cy5-labeled hsa-miR-378a-5p oligonucleotide and 2'-O-methyl modified and IRD-800 labeled GZMB mRNA oligonucleotide. Lanes 1 and 2 show the mobility of the labeled mRNA or microRNA oligonucleotide. Lane 3 shows the mobility of the labeled hsa-miR-378a-5p oligonucleotide with GZMB mRNA oligonucleotide. Lanes 4 and 6 show the mobility of labeled GZMB mRNA oligonucleotide in presence of unlabeled excess specific competitor (hsa-miR-378a-5p) and excess unlabeled non-specific competitor (NC)


HL-60
% Cell Suvival
Relative Caspase activity
Cytarabine Concentration (μM)
AML-193
% Cell Suvival
Relative Caspase activity
Cytarabine Concentration (μM)
THP-1
% Cell Suvival
Relative Caspase activity
Cytarabine Concentration (μM)
MV-4-11
% Cell Suvival
Relative Caspase activity
Cytarabine Concentration (μM)
KG-1
% Cell Suvival
Relative Caspase activity
Cytarabine Concentration (μM)
MOLM-16
% Cell Suvival
Relative Caspase activity
Cytarabine Concentration (μM)
ME-1
% Cell Suvival
Relative Caspase activity
Cytarabine Concentration (μM)
Kasumi-1
% Cell Suvival
Relative Caspase activity
Cytarabine Concentration (μM)

Supplementary Figure 2.1. Characterization of AML Cell lines for Cytarabine chemosensitivity. AML cell lines (n = 8) were cultured in the respective media and standard culturing conditions. Cytarabine-induced cytotoxicity (blue line) was determined by MTT assay after 48h of drug treatment and AUC was calculated using the cell survival data. Cytarabine-induced apoptosis (red line) was determined using Caspase 3/7 assay after 48h of drug treatment and relative caspase AUC was calculated.



Supplementary Figure 2.2: miRNA and cytarabine cell cytoxicity. Correlation plots showing association of selective miRNAs with cell survival AUC post cytarabine treatment of 8 AML cell lines.



Supplementary Figure 2.3: Network of microRNAs and important biological processes. MiRNAs predictive of in vitro cytarabine chemosensitivity in cell lines as well as with OS in AML patients were analyzed using Ingenuity pathway analysis tools and pathways identified are depicted in the network.

Table 2.1: Characterization of AML Cell lines for Cytarabine Chemosensitivity

Cell Lines	Cytogenetics /Molecular Abnormality	Cytarabine Cytotoxicity AUC (± SD)	Cytarabine Apoptosis AUC (± SD)
Kasumi-1	t(8;21)(q22;q22) → RUNX1/AML1-RUNX1T1/ETO fusion gene; TP53 mutant gene	14713 (± 582)	409.3 (± 20)
THP-1	t(9;11)(p21;q23) → MLL-AF9 fusion gene; CDKN2A, KDM6A, NRAS mutant genes	17170 (± 1341)	1544.5 (± 2)
MOLM-16	t(6;8)(q21;q24.3) and t(9;18)(q13;q21)	12021 (± 480)	490.2 (± 18)
AML-193	+der(17)t(17;17)(p13.1;q21.3)	12988 (± 366)	852.4 (± 34)
MV-4-11	FLT3 ITD mutation, t(4;11)(q21;q23) → MLL-AF4 fusion gene	5011 (± 442)	458.4 (± 22)
ME-1	inv(16)(p13q22) → CBFB-MYH11 fusion gene	6497 (± 280)	753.6 (± 33)
KG-1	NRAS mutation, P53 mutation, RB1 rearrangement	5939 (± 464)	472.5 (± 16)
HL-60	CDKN2A, NRAS, TP53 mutant genes	4597 (± 397)	555.6 (± 29)

Table 2.2: MicroRNAs significantly associated with cytarabine-induced cytotoxicity AUC and cytarabine-induced apoptosis (Caspase 3/7 activity).

MicroRNA	Spearman r	P value
Cytarabine-induced cell cytotoxicity AUC		
hsa-miR-107	0.7619	0.028
hsa-miR-148b-3p	0.7381	0.037
hsa-miR-155-5p	-0.8095	0.015
hsa-miR-16-5p	-0.7381	0.037
hsa-miR-196a-5p	-0.7857	0.021
hsa-miR-24-3p	-0.8095	0.015
hsa-miR-25-3p	0.7857	0.021
hsa-miR-374a-5p	0.7381	0.037
hsa-miR-425-5p	0.7619	0.028
Cytarabine-induced Apoptosis AUC		
hsa-miR-10a-5p	0.7857	0.021
hsa-miR-197-3p	-0.8571	0.007
hsa-miR-27b-3p	-0.7186	0.045
hsa-miR-29a-3p	0.8810	0.004
hsa-miR-29b-3p	0.7857	0.021
hsa-miR-30e-5p	0.7381	0.037
hsa-miR-324-5p	-0.9048	0.002
hsa-miR-33a-5p	0.8095	0.015
hsa-miR-378a-3p	0.8095	0.015
hsa-miR-378g	0.7381	0.037
hsa-miR-421	-0.7619	0.028

Table 2.3: MiRNAs-mRNA pairs predictive of overall survival (OS) in AML Patients (data from TCGA)

miRNA-mRNA Pair				**miRNA-mRNA correlation**		**mRNA-risk group**		**mRNA-OS**		
miRNA	mRNA probe	mRNA gene	mRNA Chr	Spearman Correlation		Kruskal-Wallis		Unstratified Cox Model		
				r	p value	p value	FDR	HR	p value	FDR
mir-107	212202_s_at	TMEM87A	chr15q15.1	-0.208	0.004	0.000	0.001	0.386	0.000	0.045
mir-107	204798_at	MYB	chr6q23.3	-0.235	0.001	0.050	0.075	0.430	0.000	0.004
mir-10a	201724_s_at	GALNT1	chr18q12.2	-0.418	0.000	0.000	0.000	0.327	0.000	0.006
mir-10a	218181_s_at	MAP4K4	chr2q11.2	-0.445	0.000	0.046	0.072	0.558	0.001	0.045
mir-10a	222273_at	PAPOLG	chr2p16.1	-0.174	0.018	0.429	0.350	0.392	0.000	0.013
mir-148b	218181_s_at	MAP4K4	chr2q11.2	0.240	0.001	0.046	0.072	0.558	0.001	0.045
mir-16	213150_at	HOXA10*	chr7p15.2	0.441	0.000	0.000	0.000	1.216	0.000	0.041
mir-16	201724_s_at	GALNT1	chr18q12.2	-0.217	0.003	0.000	0.000	0.327	0.000	0.006
mir-16	212314_at	SEL1L3	chr4p15.2	0.263	0.000	0.000	0.000	1.446	0.001	0.046
mir-16	209193_at	PIM1	chr6p21.2	0.247	0.001	0.002	0.006	1.713	0.000	0.045
mir-196a	214651_s_at	HOXA9*	chr7p15.2	0.293	0.000	0.000	0.000	1.166	0.001	0.046
mir-196a	204779_s_at	HOXB7	chr17q21.32	0.223	0.002	0.000	0.000	1.276	0.001	0.046
mir-196a	201852_x_at	COL3A1	chr2q32.2	0.252	0.001	0.152	0.170	0.783	0.000	0.045
mir-196a	213687_s_at	RPL35A	chr 3q29	-0.174	0.018	0.300	0.276	0.399	0.000	0.042
mir-197	212202_s_at	TMEM87A	chr15q15.1	-0.184	0.012	0.000	0.001	0.386	0.000	0.045
mir-197	218181_s_at	MAP4K4	chr2q11.2	0.159	0.030	0.046	0.072	0.558	0.001	0.045
mir-29b	207106_s_at	LTK*	chr15q15.1	-0.184	0.012	0.000	0.000	0.790	0.001	0.046

Table 2.3 (Continued): MiRNAs-mRNA pairs predictive of overall survival (OS) in AML Patients (data from TCGA)

miRNA-mRNA Pair				**miRNA-mRNA correlation**		**mRNA-risk group**		**mRNA-OS**		
miRNA	mRNA probe	mRNA gene	mRNA Chr	Spearman Correlation		Kruskal-Wallis		Unstratified Cox Model		
				r	p value	p value	FDR	HR	p value	FDR
mir-29b	56919_at	WDR48	chr3p22.2	-0.169	0.021	0.068	0.095	0.514	0.000	0.009
mir-30e	204779_s_at	HOXB7	chr17q21.32	-0.325	0.000	0.000	0.000	1.276	0.001	0.046
mir-30e	201724_s_at	GALNT1	chr18q12.2	0.208	0.004	0.000	0.000	0.327	0.000	0.006
mir-30e	218313_s_at	GALNT7	chr4q34.1	0.329	0.000	0.106	0.132	0.417	0.000	0.024
mir-378	210164_at	GZMB	chr14q12	-0.205	0.005	0.199	0.207	1.439	0.001	0.046
mir-421	214651_s_at	HOXA9*	chr7p15.2	-0.277	0.000	0.000	0.000	1.166	0.001	0.046

* Except for HOXA9, HOXA10 and LTK all other genes were also significantly associated with OS ($p<0.05$) in risk stratified analysis. FDR was estimated by Pounds and Cheng's robust FDR method.

Supplementary Table 2.1: Association of miRNA target genes with Overall Survival (Risk stratified and non risk stratified)

microRNA	miR-Risk group (P value)	miR-Risk group (BH95q)	miR-OS (Hazard Ratio)	miR-OS (P value)	miR-OS (BH95q)	miR-OS Risk stratified (HR)	miR-OS Risk stratified (P value)	miR-OS Risk stratified (BH95q)
hsa-mir-10a	**1.693E-12**	0.0000	1.1440	**0.0030**	0.0208	1.0860	0.1121	0.2615
hsa-mir-16	**2.163E-07**	0.0000	1.5620	**0.0114**	0.0481	1.1940	0.3617	0.5843
hsa-mir-196a	**7.793E-06**	0.0001	1.2210	**0.0029**	0.0208	1.1580	**0.0271**	0.1209
hsa-mir-197	**0.0004**	0.0019	1.3870	0.1687	0.2952	1.2030	0.4507	0.6761
hsa-mir-421	**0.0006**	0.0025	0.8890	0.2158	0.3211	0.9500	0.6267	0.7223
hsa-mir-155	**0.0130**	0.0391	1.4390	**0.0019**	0.0208	1.3120	**0.0288**	0.1209
hsa-mir-24-2	**0.0277**	0.0727	1.6250	**0.0181**	0.0544	1.4740	0.0801	0.2103
hsa-mir-374a	0.0829	0.1934	1.0020	0.9907	0.9907	1.1180	0.5264	0.6910
hsa-mir-148b	0.1040	0.2183	1.0660	0.7502	0.8753	1.2100	0.3570	0.5843
hsa-mir-107	0.1259	0.2305	1.5550	0.0966	0.2253	2.0430	**0.0085**	0.1209
hsa-mir-29a	0.1317	0.2305	1.2390	0.2294	0.3211	1.3770	0.0698	0.2095
hsa-mir-378	0.1697	0.2741	1.2470	0.1453	0.2849	1.2570	0.1622	0.3407
hsa-mir-324	0.2036	0.2850	1.1010	0.6380	0.7881	1.1550	0.5000	0.6910
hsa-mir-425	0.2269	0.2978	0.8670	0.2572	0.3375	0.9620	0.7708	0.7708
hsa-mir-30e	0.3016	0.3726	1.0350	0.9061	0.9703	0.9080	0.7395	0.7708
hsa-mir-29b	0.3613	0.4215	1.6630	**0.0077**	0.0407	1.5690	**0.0176**	0.1209
hsa-mir-25	0.4188	0.4481	0.6370	**0.0373**	0.0979	0.6210	**0.0359**	0.1258
hsa-mir-27b	0.7951	0.7951	0.7330	0.1950	0.3150	0.7370	0.1911	0.3648

P values < 0.05 are shaded and bold

CHAPTER III

MicroRNAs Mediated Regulation of Expression of Nucleoside Analog Pathway Genes

3.1. Introduction:

Nucleoside analogs (NA) are a class of chemotherapeutic agents that structurally resemble the endogenous purine or pyrimidine nucleosides. These therapeutic agents mimic the endogenous nucleosides with respect to their uptake and metabolism and are incorporated into the newly synthesized DNA leading to inhibition of DNA synthesis and chain termination. Some of the nucleoside analogs also inhibit or block the enzymes that are required for the synthesis of purine or pyrimidine nucleotides and RNA synthesis, leading to the activation of the caspase cascade and cell death. The nucleoside analogs are extensively used for the treatment of both, hematological malignancies and solid tumors. The pyrimidine nucleoside analog, cytarabine, is one of the most widely used chemotherapeutic drugs for the treatment of acute myeloid leukemia (AML). While clofarabine, a purine nucleoside analog is one of the newer nucleoside analogs used for the treatment of AML.

One of the major obstacles in the treatment of AML is development of resistance to nucleoside analogs. There is a growing need to understand the mechanisms that lead to development of resistance to these nucleoside analogs in order to help identify strategies that would effectively treat patients with relapsing or refractory disease. One of the primary mechanisms of resistance to nucleoside analogs is insufficient intracellular concentration of the active triphosphate metabolite. This insufficient triphosphate levels could be due to inefficient cellular uptake of the drug, reduced levels of the activating enzyme, increased levels of inactivating enzymes and/or due to increased levels of endogenous deoxynucleotide (dNTP) pools [1-4]. Resistance could also develop due to

inability to achieve sufficient alterations in the DNA strands or the dNTP pools, either due to altered interaction with DNA polymerases or by lack of inhibition of ribonucleotide reductases, or due to inadequate p53 exonuclease activity. Since the expression and activity of drug transporters and metabolizing enzymes in the activation pathway of nucleoside analogs plays an important role in development of resistance to the NAs, it is essential to understand the factors influencing the expression and activity of these proteins.

MicroRNAs are a group of novel gene regulators, which have been recently recognized to play an important role in cancers due to their tumor suppressive and oncogenic functions [5]. MicroRNAs are known to regulate the expression of the target genes by binding to the specific sequence on the 3' untranslated region on the genes. Role of microRNAs in regulating the expression of various drug metabolizing enzymes like CYP3A4 etc., drug transporters like BCRP and various drug targets [6-8] has been established. However, comprehensively evaluation of the effect of microRNAs on the genes involved in the transport, activation and inactivation genes of nucleoside analogs has not been done. Hence, the aim of this study was to assess the effect of microRNAs on the expression of nucleoside analog PK and PD pathway genes and in turn assessing their potential impact on resistance to nucleoside analogs.

3.2. Materials and Methods

3.2.1. Cell culture and Reagents: The AML cell lines HL-60, MV-4-11, Kasumi-1, THP-1, AML-193 and KG-1 cell lines were obtained from American Type Culture

Collection (ATCC) (Manassas, VA), while MOLM-16 and ME-1 cell lines were obtained from DSMZ (Braunschweig, Germany). Kasumi-1, ME-1 and MOLM-16 cell lines were cultured in RMPI-1640 medium supplemented with 20% fetal bovine serum (FBS), THP-1 cell line was cultured in RPMI-1640 medium supplemented with 10% FBS, MV-4-11 cell line was cultured in IMDM medium supplemented 10% FBS, HL-60 and KG-1 cell lines were cultured in IMDM medium supplemented with 20% FBS, while AML-193 cell lines was cultured in IMDM medium supplemented with 5% FBS, 0.005 mg/ml insulin, 0.005 mg/ml transferrin and 5 ng/ml GM-CSF. All the cell lines were maintained in a 37°C humidified incubator with 5% CO_2. The cells were passaged every 2 to 3 days in order to maintain them in logarithmic growth phase.

3.2.2. RNA Isolation: Total RNA was isolated from the AML cell pellets using RNeasy Plus Mini Kit (QIAGEN, USA) according to the manufacturer's protocol and stored in -80°C until further analysis. The RNA quality and concentration was measured using NanoDrop 2000 UV-Vis spectrophotometer (ThermoScientific, USA). The ratio of absorbance at 260 nm and 280 nm was used to assess RNA sample purity and A260/A280 ratio of 1.8-2.1 was considered to be indicative of highly purified RNA. RNA was normalized to 0.2µg/µl with nuclease-free water before being used for performing reverse transcription reactions, as recommended by the manufacturer. The total RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to manufacturer's protocol.

3.2.3. Gene Expression Analysis: The expression of nucleoside analog genes was determined using the TaqMan® Low Density Array (TLDA) cards (Applied Biosystems,

USA). Each TLDA card was custom designed with pre-loaded gene expression assays for measuring the mRNA expression of selected nucleoside analog metabolic pathway genes-(n=14) deoxycytidine kinase (DCK), cytidine deaminase (CDA), solute carrier family 29, member 1 (SLC29A1), solute carrier family 28, member 1 (SLC28A1), solute carrier family 28, member 3 (SLC28A3), deoxycytidylate deaminase (DCTD), 5'-nucleotidase, cytosolic II (NT5C2), 5'-nucleotidase cytosolic III (NT5C3), cytidine 5'-triphosphate synthase (CTPS), cytidine monophosphate kinase (CMPK), nucleoside diphosphate kinase 1 (NME1), ribonucleotide reductase M1 (RRM1), ribonucleotide reductase M2 (RRM2), ribonucleotide reductase M2B (RRM2B). Each TLDA card consists of eight separate loading ports that fill into 48 separate wells, for a total of 384 wells per card. Thus, each card could analyze the expression of 24 different genes for 8 different samples in duplicates. Each cDNA sample was added to equal volume of 2X TaqMan Universal PCR Master Mix and 100 µl of the sample-specific PCR mix was added to the fill reservoir on the TLDA card. The card was centrifuged twice for 1 minute at 1200 rpm to distribute the sample-specific PCR reaction mix to the reaction wells. The card was sealed using the TaqMan Array Micro Fluidic Card Sealer and placed on microfluidic card thermal cycling block of Applied Biosystems 7900HT Fast Real-time PCR System (Applied Biosystems). Thermal cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 94.5°C, 30 seconds at 97°C, 1 minute at 59.7°C for 40 cycles. The target mRNA expression levels were normalized to GAPDH and the expression values of nucleoside analogs pathway genes were calculated using comparative C_T method [9].

3.2.4. MicroRNA Expression Analysis: For determination of microRNA expression, total RNA was isolated using mirVana™ miRNA Isolation kit (Life Technologies, USA) as per the manufacturer's protocol. The RNA quality and concentration was measured using NanoDrop 2000 UV-Vis spectrophotometer (ThermoScientific, USA). A total of 100 ng of purified total RNA was used for nCounter miRNA sample preparation reactions according to manufacturer's instructions and was assayed for determination of 800 human microRNA expression using the nCounter Human v2 miRNA Expression Assay kit (Nanostring Technologies, USA). Preparation of small RNA samples involved multiplexed ligation of specific tags (miRtags) to the target microRNAs that provide unique identification for each microRNA species. After ligation, the detection was done by hybridization to microRNA: tag specific nCounter capture and barcoded reporter probes. Data collection was carried out using the nCounter Digital Analyzer (Nanostring Technologies, USA) at The University of Minnesota Genomics Center, following manufacturer's instructions to count individual fluorescent barcodes and quantify the target microRNA molecules present in each sample. MicroRNA expression data normalization was performed using the nSolver™ Analysis Software (Nanostring Technologies) according to the manufacturer's instructions. In particular, initially the data was normalization using the expression of the top 100 codesets was used. Further, to account for the background correction, mean of negative controls plus two-standard deviation (SD) method was used. In order to avoid using the microRNAs with a very low expression, we further filtered out the microRNAs with expression counts <30 (2 times

the mean ± 2 SD of negative control value), in order to account for the background noise. Total 412 microRNAs with expression counts >30 were included for further analysis.

3.2.5. AML Patient Sample Data from The Cancer Genome Atlas (TCGA): The microRNA expression and mRNA expression of the nucleoside analog pathway genes in AML patients was extracted from The Cancer Genome Atlas (TCGA) Data Portal (cancergenome.nih.gov) [10]. Out of the 200 AML patients in TCGA database, 197 patients had gene expression profiling data available and 187 patients had microRNA expression data available. 186 patients had both gene expression and microRNA expression data available.

3.2.6. Electrophoretic Mobility Shift Assays: The functional validation for binding efficiencies between microRNAs and mRNAs was performed using the electrophoretic mobility shift assays (EMSAs). The binding free energy between the respective mRNA and microRNA pair was predicted using the RNAhybrid software. The microRNA oligonucleotides were labeled with cy5™ dye on their 5' ends. The 2' O-methyl-modified mRNA oligonucleotides were labeled with IRDye®800 (LI-COR Biosciences, USA) dye on their 5' ends. The labeled oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, USA). RNA EMSA experiment was performed using the LightShift Chemiluminescent RNA EMSA Kit (ThermoScientific, USA) according to the manufacturer's protocol. The mRNA oligonucleotide was heated for 10 minutes at 80°C and then placed on ice in order to relax the secondary structures. In each 20µl binding reaction, 200nM microRNA oligonucleotide and /or mRNA oligonucleotide were mixed with RNA EMSA binding buffer and incubated at 25°C for 25 minutes. The reaction

mixtures were separated on a 12% polyacrylamide gel by electrophoresis at 4°C. The binding reactions were electrophoretically transferred onto nylon membrane and the resulting mobility shifts were imaged using and Odyssey CLx Infrared System (LI-COR Biosciences, USA).

3.2.7. Bioinformatic Analysis: Prediction of microRNA binding sites was performed using multiple prediction programs, which use different criteria for prediction of binding sites: TargetScan (www.targetscan.org), miRanda (www.microRNA.org), PICTAR (pictar.mdc-berlin.de), miRWalk (www.umm.uni-heidelberg.de/apps/zmf/mirwalk). Binding free energy calculations were performed using RNAhybrid software. The 3'UTR sequence of mRNA was obtained from the UCSC Genome browser and microRNA sequence was obtained from miRBase software.

3.2.8. Statistical Analysis: The nonparametric Spearman correlation was used to measure the correlation of mRNA expression with microRNA expression. Statistical significance was determined when p-value was < 0.01.

3.3. Results

3.3.1. Effect of microRNA on the expression of nucleoside analog pathway genes in AML Cell lines: We determined expression of 800 microRNAs and 13 genes involved in PK/PD pathway of nucleoside analogs (Figure 3.1) in cytogenetically different AML cell lines ($n = 8$). In order to identify the microRNAs associated with the expression of nucleoside analog pathway genes, we correlated the microRNA expression and mRNA expression using the spearman correlation analysis. Table 3.1 lists the correlation of

nucleoside analog pathway genes and respective microRNAs at p <0.01. Figure 3.2 depicts the network of microRNAs correlated with the respective PK/PD pathway genes of the nucleoside analogs.

The expression of DCK (the rate-limiting enzyme in the nucleoside analog pathway) correlated with the expression of miR-34a-5p expression (spearman r = -0.88; p-value < 0.01) and miR-96-5p expression (spearman r = -0.91; p-value < 0.01). The expression of deactivating enzyme DCTD was found to be correlated with miR-24-3p expression (spearman r = -0.93; p-value < 0.01). Interestingly, in our previous study (Chapter II) we identified that expression of microRNA miR-24-3p was correlated with cytarabine-induced cell cytotoxicity (spearman r = -0.81, p-value < 0.05) which is in agreement with the current observation. The expression of CMPK, a kinase responsible for phosphorylation of the monophosphate form of nucleoside analog was negatively correlated with the expression of miR-1301, miR-1323, miR-320e, miR-381, miR-507, miR-584-5p, miR-605, miR-762, miR-769-3p, miR-891a (all p-values < 0.01). RRM2 expression was found to be negatively associated the expression of miR-151a-3p (p-value < 0.01).

3.3.2. Bioinformatic prediction of binding of microRNAs and mRNAs: MicroRNAs are known to guide the RNA-induced silencing complex (RISC) to the specific sequence (usually in located in the 3'UTR) on the target mRNA. Using various bioinformatic prediction programs (TargetScan, miRanda, PICTAR, miRWalk) we determined if the microRNAs that were correlated with gene expression had binding sites on 3'-UTR of target genes. MicroRNAs miR-1323, miR-30d-5p, miR-381, and miR-605 were

predicted to have binding sites on CMPK gene, while microRNA miR-24-3p was found to have binding site on DCTD by multiple prediction programs. Additionally, miR-181-5p, let-7g-5p and miR194-5p were also found to have binding sites on CTPS1, RRM2 and RRM2B genes, respectively.

3.3.3. Effect of microRNAs on the expression of nucleoside analog pathway genes in AML patients: In order to validate the significant correlations between microRNAs and mRNAs identified in AML cell lines, we evaluated the correlation between microRNA expression and nucleoside analog pathway gene expression in AML patient samples from TCGA database (n=186). We extracted the microRNA expression data and nucleoside analog pathway gene expression data for AML patients from TCGA database and performed spearman correlation to identify the significant mRNA-microRNA pairs. Table 3.2 lists the mRNA-microRNA correlations, which were common in AML cell lines and AML patient samples from TCGA database. MicroRNA miR-24-3p was inversely correlated with the expression of both the probes for DCTD in AML patient samples ($r = -0.22$; p-value < 0.01 and $r = -0.21$; p-value < 0.01). In addition, miR-23a, miR-181a, miR-149, miR-140 were also found to be significantly correlated with expression of CDA, CTPS, NT5C3 and RRM2, respectively, in AML patients. (Table 3.2).

3.3.4. Validation of binding efficiencies between mRNAs and microRNAs: MicroRNAs are known to bind to the specific seed sequence on the 3'UTR of the mRNAs, thereby regulating the expression of their target genes. In order to validate the binding between the mRNA-microRNAs identified from the *in vitro* studies and in AML

patient samples, we performed RNA EMSA assays. We decided to validate the interaction between DCTD and miR-24, since we identified this mRNA-microRNA pair to be significantly inversely correlated in both AML cell line and AML patient samples. In addition, various prediction databases predicted microRNA miR-24 to have binding site on the 3'UTR of DCTD mRNA. We also decided to validate the interaction between DCK and miR-34a. *In silico* analysis predicted miR-24-3p and miR-34a-5p might form complexes with target sequences in the 3'UTR of DCTD and DCK respectively with minimum free energies of binding of -27.2 kcal/mol for DCTD and miR-24-3p (Figure 3.4A) and -25.6 kcal/mol for DCK and miR-34a-5p (Figure 3.5A). The RNA EMSA results for IRD-800 ®-labeled DCTD and Cy-5-labeled miR-24-3p show miR-24a-3p was able to bind to its target sequence on DCTD 3'UTR (Figure 3.4B, lane 3) as seen by the band shift. The thermodynamic stability of this complex correlated with binding observed in the RNA EMSA assays. In addition, we found that mRNA-microRNA complex formed by DCTD and miR-24a-3p could be eliminated by adding excess unlabeled hsa-miR-24a-3p probe (Figure 3.4B, lane 4), but not by adding excess unlabeled non-specific probe (Figure 3.4B, lane 6). Adding excess unlabeled mRNA probe resulted in binding of all the labeled microRNA giving a greater intensity signal (Figures 3.4B, lane 5).

Similar to the interaction observed between DCTD and miR-24a-3p, we observed that miR-34a-5p binds to DCK 3'UTR as seen by shift in the band (Figure 3.5B, lane 3) and this interaction was eliminated by addition of unlabeled probe (Figure 3.5B, lane 4).

3.4. Discussion

Nucleoside analogs are synthetic analogs of endogenous nucleosides that largely used for the treatment of hematological malignancies and solid tumors. Cytarabine, a pyrimidine nucleoside analog is the backbone of AML chemotherapy, while a second-generation purine nucleoside analog, clofarabine is currently being investigated for treatment of AML in various clinical trials. Both cytarabine and clofarabine require active transport into the cell by nucleoside transporters, followed by activation by various kinases to form di- and tri-phosphate metabolites that are incorporated in growing DNA strand and/or inhibit various enzymes involved in synthesis of endogenous nucleotides (Figure 3.1). However, despite being the backbone of treatment regimen used in AML patients for several decades, there is wide variation in AML patients in response to cytarabine. In our previous study (Chapter II), we have demonstrated that microRNA expression is predictive of response to cytarabine therapy in AML patients and is also significantly associated with *in vitro* chemosensitivity of cytarabine in AML cell lines. In the current chapter, we wanted to determine if microRNA mediated regulation of genes involved in cytarabine transporter, activation/inactivation could contribute to development of resistance in AML. We hypothesized that microRNAs could bind to the 3'UTR of the cytarabine metabolic pathway genes, thereby altering their expression, which in turn would result in alterations in intracellular levels of active NA triphosphate, resulting in chemoresistance. To the best of our knowledge, this is the first study that has determined the effect of microRNA on the expression of the entire metabolic pathway of nucleoside analogs.

In our current study, DCK expression was negatively correlated with the expression of miR-34a-5p and miR-96-5p in AML cell lines (p-value < 0.01). DCK is a rate-limiting enzyme that is involved in activation of cytarabine, clofarabine and other nucleoside analogs. Studies have reported that decreased or complete loss of DCK activity results in cellular resistance to cytarabine [11-13]. Also DCK mRNA expression has been shown to be positively associated with AML patient outcome, AML patients with higher DCK mRNA expression had longer event-free survival than those with lower DCK mRNA expression [14]. Various studied have identified multiple single nucleotide polymorphisms (SNPs) in the proximal promoter region, coding region as well as the intronic region of the DCK gene, which were associated with the DCK mRNA expression [15], or DCK activity [16]. Till date only one study has demonstrated the effect of microRNA expression on DCK, suggesting a possible role of miR-330 in the post-transcriptional regulation of DCK [17]. In the current study, using RNA EMSA, we were able to show that miR-34a-5p by binding to the 3'-UTR regulates expression of DCK. MicroRNA miR-34a has been extensively studied in various cancers [18-22] and it has been shown to play an important role as a tumor suppressor by targeting various genes. However, the effect of miR-34a on DCK expression has not yet been studied. Identification of this additional regulatory mechanism for an important enzyme in the activation of nucleoside analogs could help in better prediction of chemosensitivity of these drugs. We also identified miR-24-3p to be negatively correlated with the expression of DCTD in both AML cell lines and in AML patient samples (p-value<0.01). In addition, multiple bioinformatic prediction programs identified a binding site for hsa-

miR-24-3p on the 3'UTR of DCTD. Our RNA EMSA results confirmed the binding interaction between DCTD and miR-24-3p. DCTD is an enzyme involved in deamination of the monophosphate form of the nucleoside analog, thus inactivating the drug. The levels of DCTD could thus affect the levels of the intracellular active triphosphate metabolites of nucleoside analogs. However, the role of DCTD in chemosensitivity of nucleoside analogs is poorly defined. Various studies have demonstrated a significant role of DCTD in metabolism of the monophosphate metabolite of the nucleoside analogs in human leukemia cells [23-25]. Sequencing of this gene identified a nonsynonymous SNP affecting the activity of DCTD *in vitro* [26]. Hence, the limited data on the regulation of DCTD gene warrants identification of additional mechanism of regulation of this gene. MicroRNA miR-24 is has also been extensively studied in various cancers [27-33] and has been shown to enhance metastasis and invasion. Increased expression of miR-24 has been associated with increased risk of relapse and poor survival in ALL [31]. In addition to miR-24 and miR-34a-5p, we also identified multiple miRNAs that correlated with multiple genes in the PK/PD pathway (Table 3.1 and Table 3.2). While, these miRNAs individually did not correlate with overall survival in patients from TCGA database (Chapter II), they along with other miRNAs can potentially contribute to the development of resistance in patients treated with cytarabine or clofarabine.

In summary, we identified several microRNAs, which were significantly associated with the expression of nucleoside analog pathway genes *in vitro* as well as *in vivo*. Identification of these additional mechanisms of regulation would enhance our understanding of the variability in the expression of these enzymes and transporters and

in turn, help in better prediction of therapeutic response in AML patients. While additional functional studies are required to gain mechanistic understanding of these microRNA-mRNA interactions and its effect on the protein levels and activity, this study helps identify candidate microRNAs for further studies.

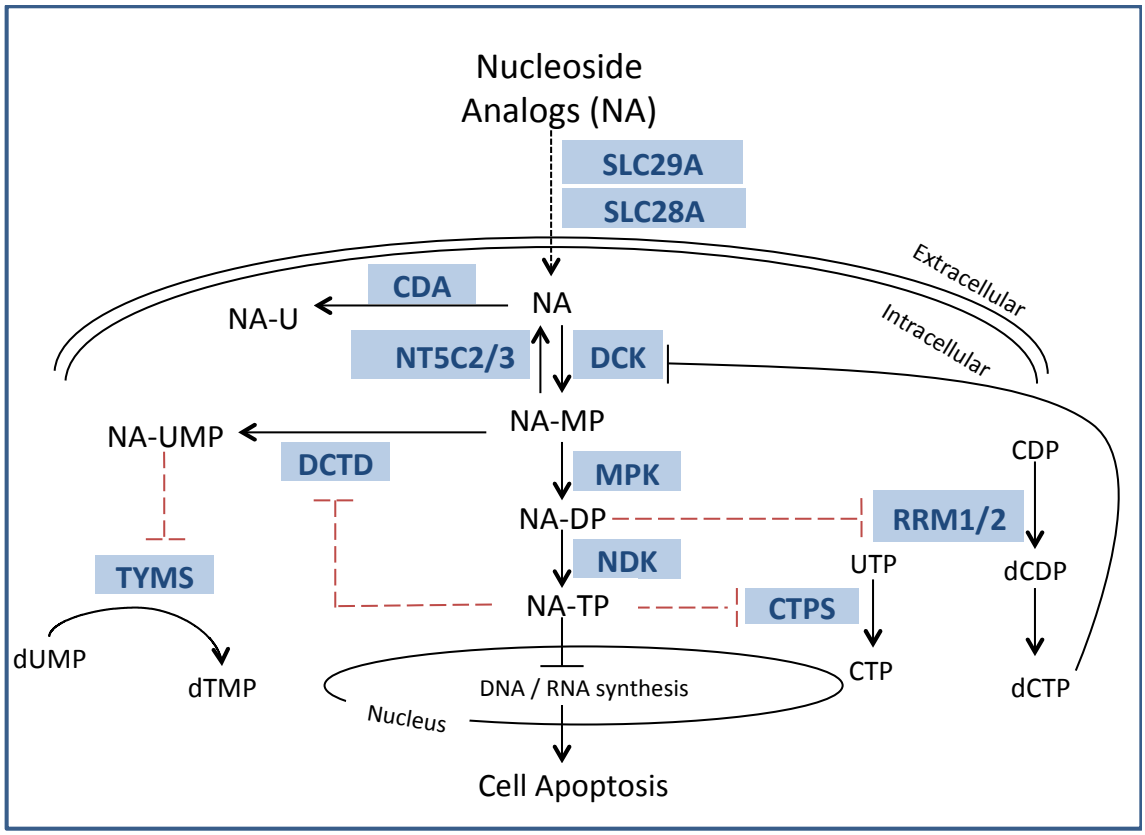


Fig 3.1: PK/PD Pathway of Nucleoside Analogs: Cytarabine and Clofarabine

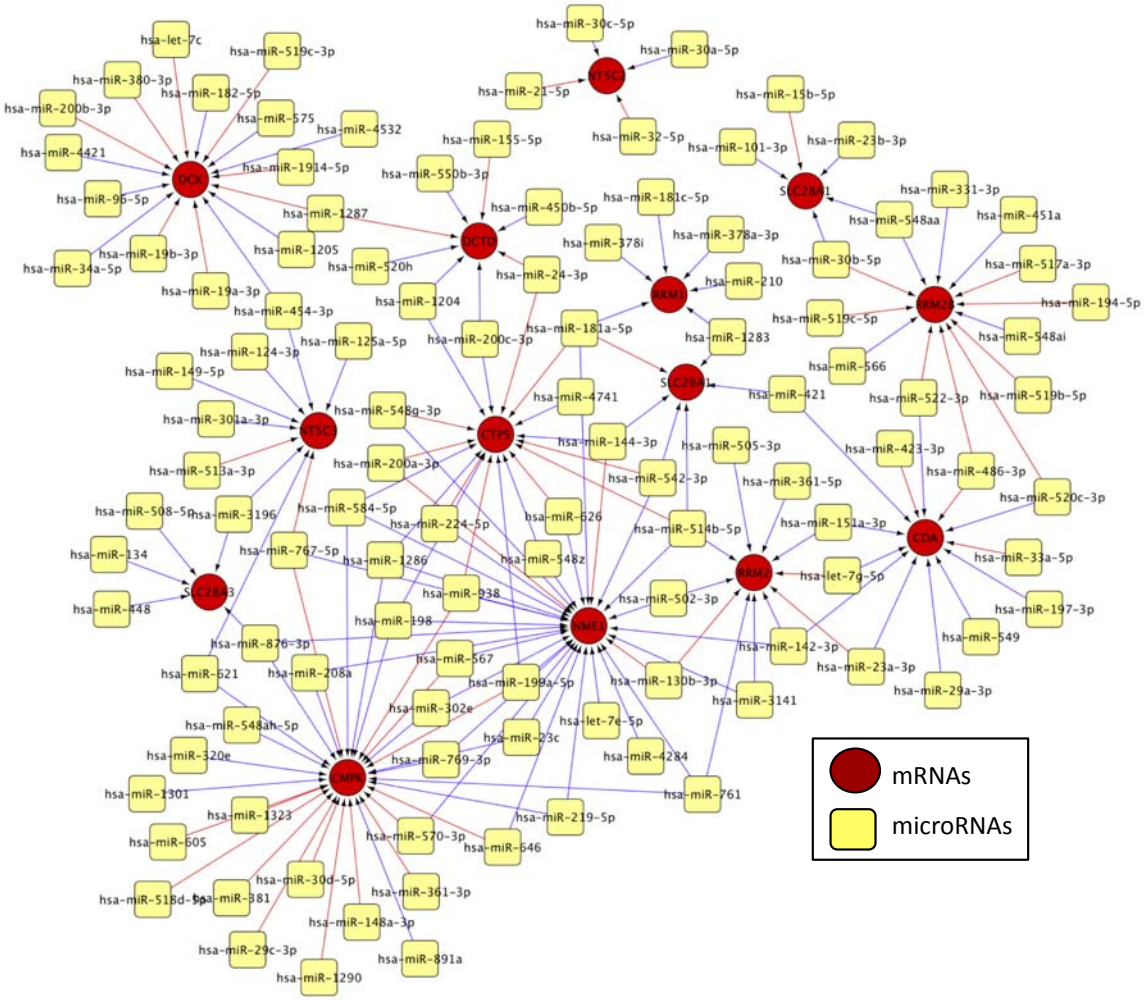
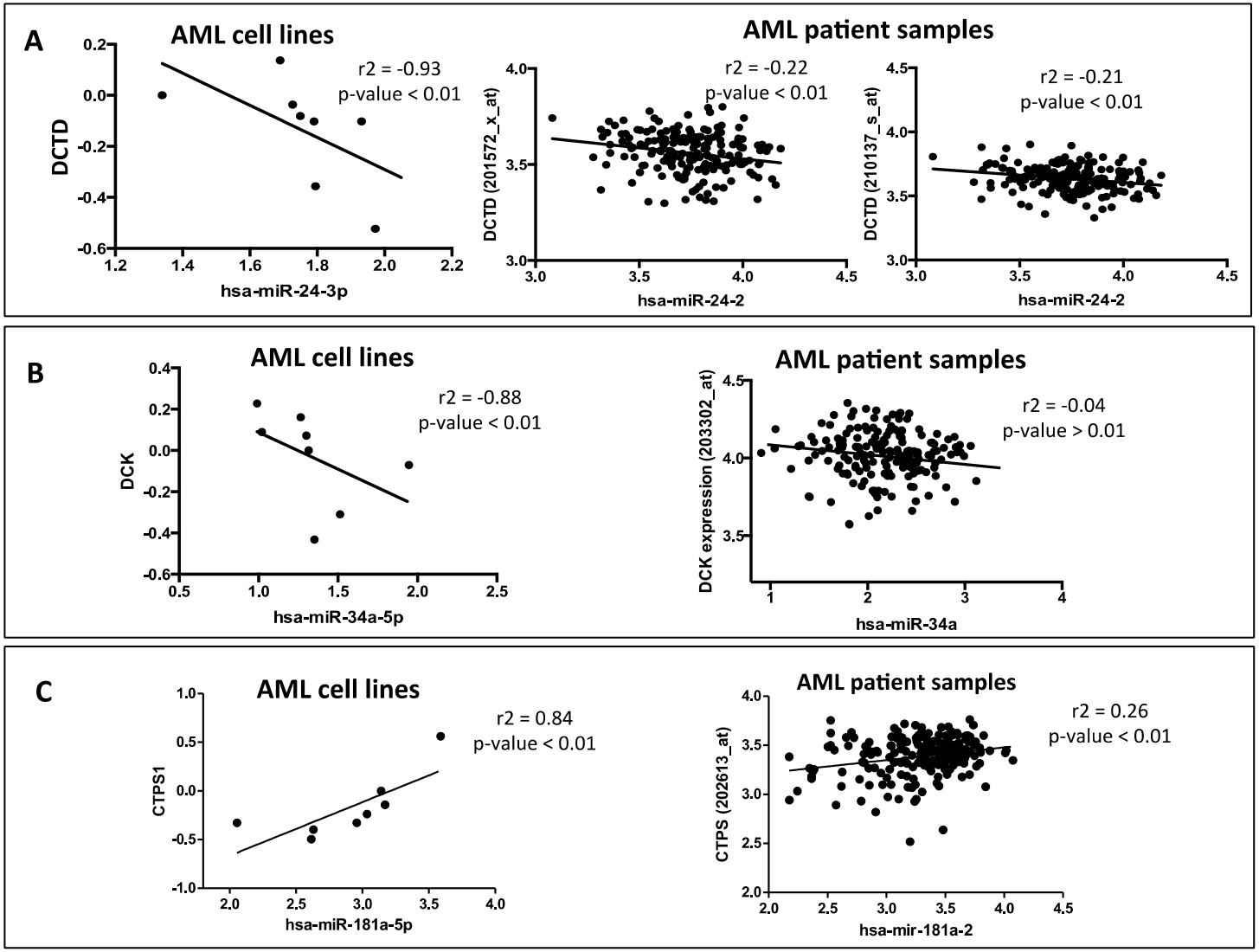


Figure 3.2: Constructing microRNA-mRNA network using CyTargetLinker.

MicroRNAs associated with the nucleoside analog metabolic pathway genes.


A
AML cell lines
r2 = -0.93
p-value < 0.01
DCTD
hsa-miR-24-3p
AML patient samples
r2 = -0.22
p-value < 0.01
DCTD (201572_x_at)
hsa-miR-24-2
r2 = -0.21
p-value < 0.01
DCTD (210137_s_at)
hsa-miR-24-2
B
AML cell lines
r2 = -0.88
p-value < 0.01
DCK
hsa-miR-34a-5p
AML patient samples
r2 = -0.04
p-value > 0.01
DCK expression (203302_at)
hsa-miR-34a
C
AML cell lines
r2 = 0.84
p-value < 0.01
CTPS1
hsa-miR-181a-5p
AML patient samples
r2 = 0.26
p-value < 0.01
CTPS (202613_at)
hsa-mir-181a-2

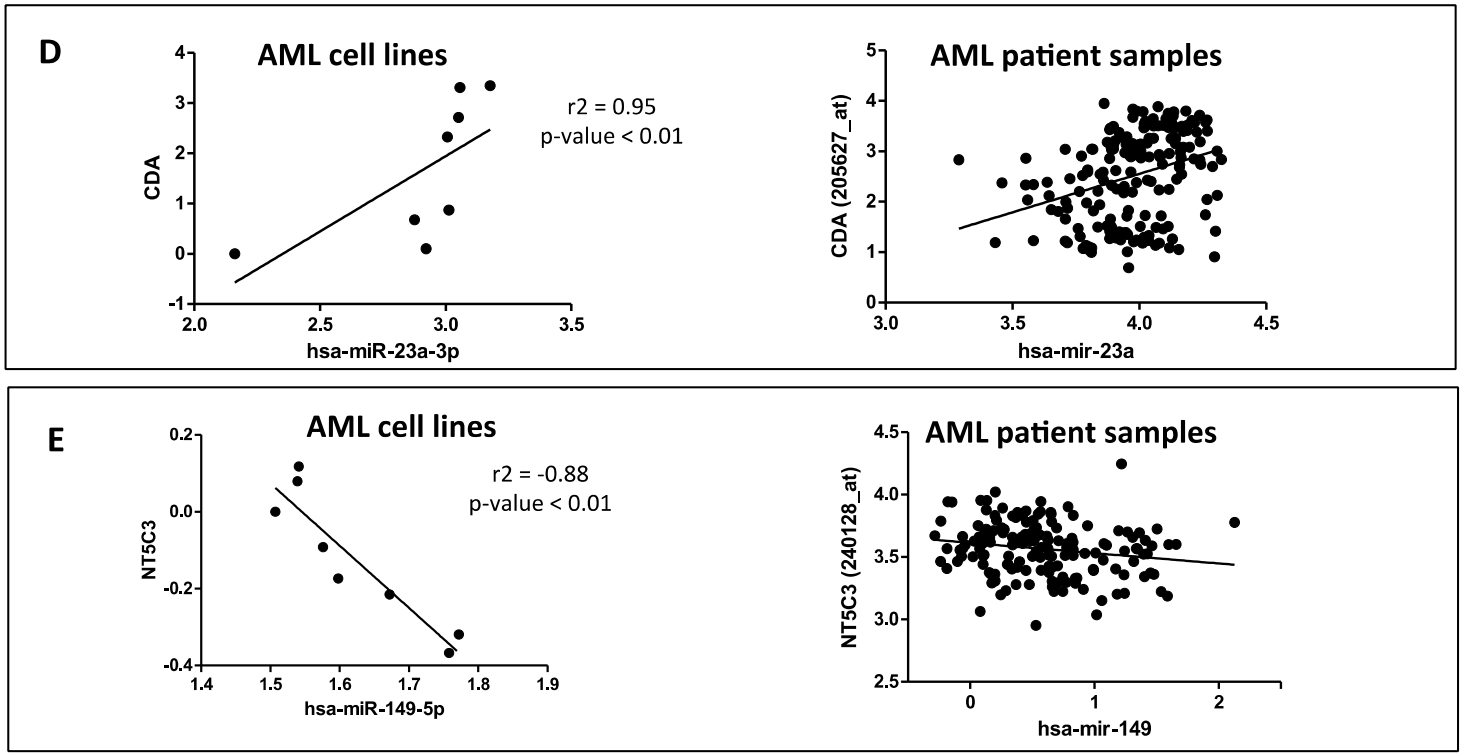


Figure 3.3: Correlation of microRNAs with nucleoside analog pathway gene expression in AML cell lines and patient samples from TCGA database. (A) Correlation between DCTD and hsa-miR-24a-3p. (B) Correlation between DCK and hsa-miR-34a-5p. (C) Correlation between CTPS1 and hsa-miR-181a. (D) Correlation between CDA and hsa-miR-23a. (E) Correlation between NT5C3 and hsa-miR-149.

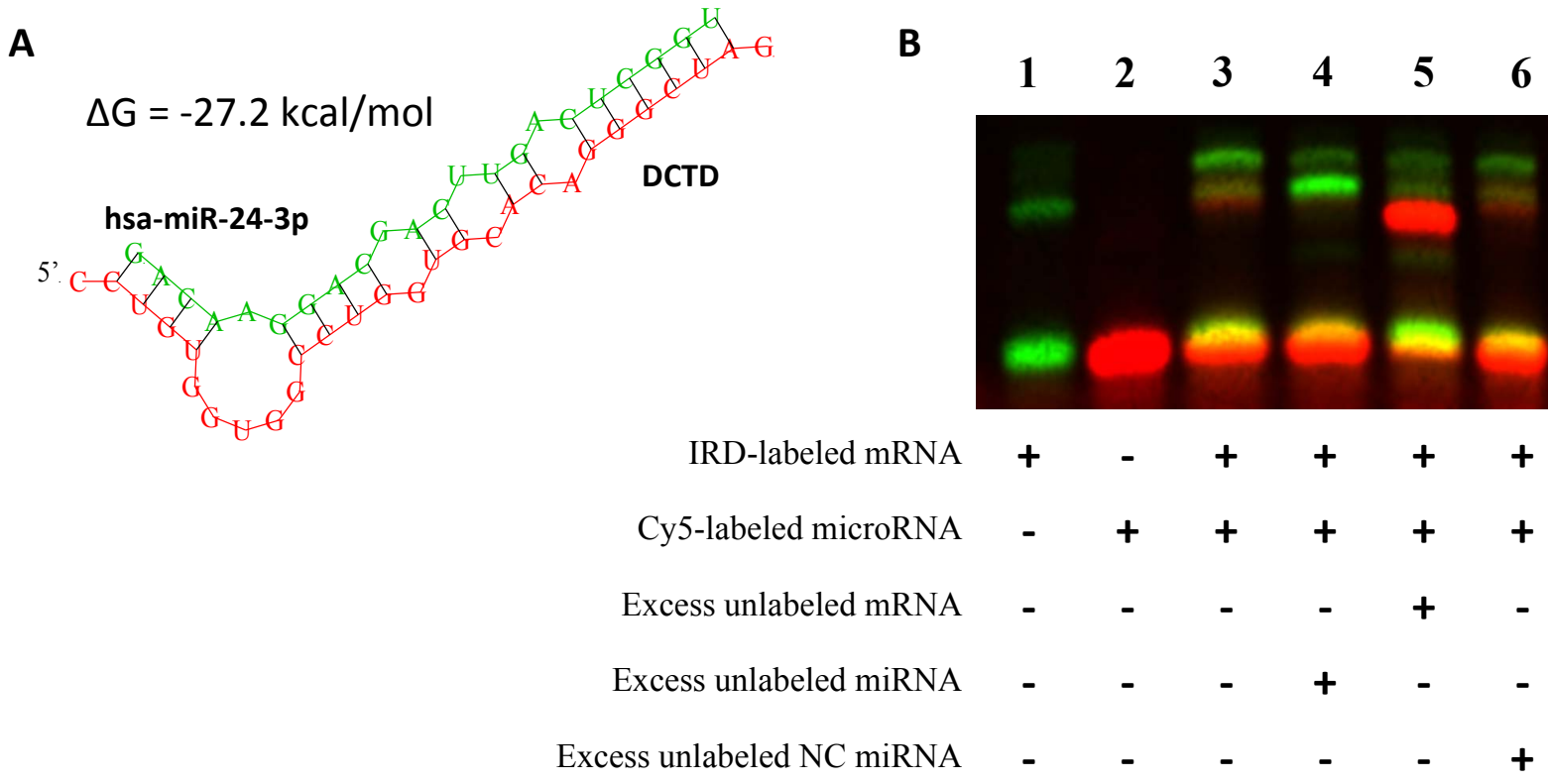


Figure 3.4: Validation of binding interaction between DCTD mRNA and hsa-miR-24-3p by RNA EMSAs. RNA EMSA with cy5-labeled hsa-miR-24-3p oligonucleotide and 2'-O-methyl modified and IRD-800 labeled DCTD mRNA oligonucleotide. Lanes 1 and 2 show the mobility of the labeled mRNA or microRNA oligonucleotide. Lane 3 shows the mobility of the labeled hsa-miR-24-3p oligonucleotide with DCTD mRNA oligonucleotide. Lanes 4 and 6 show the mobility of labeled DCTD mRNA oligonucleotide in presence of unlabeled excess specific competitor (hsa-miR-24-3p) and excess unlabeled non-specific competitor (NC)

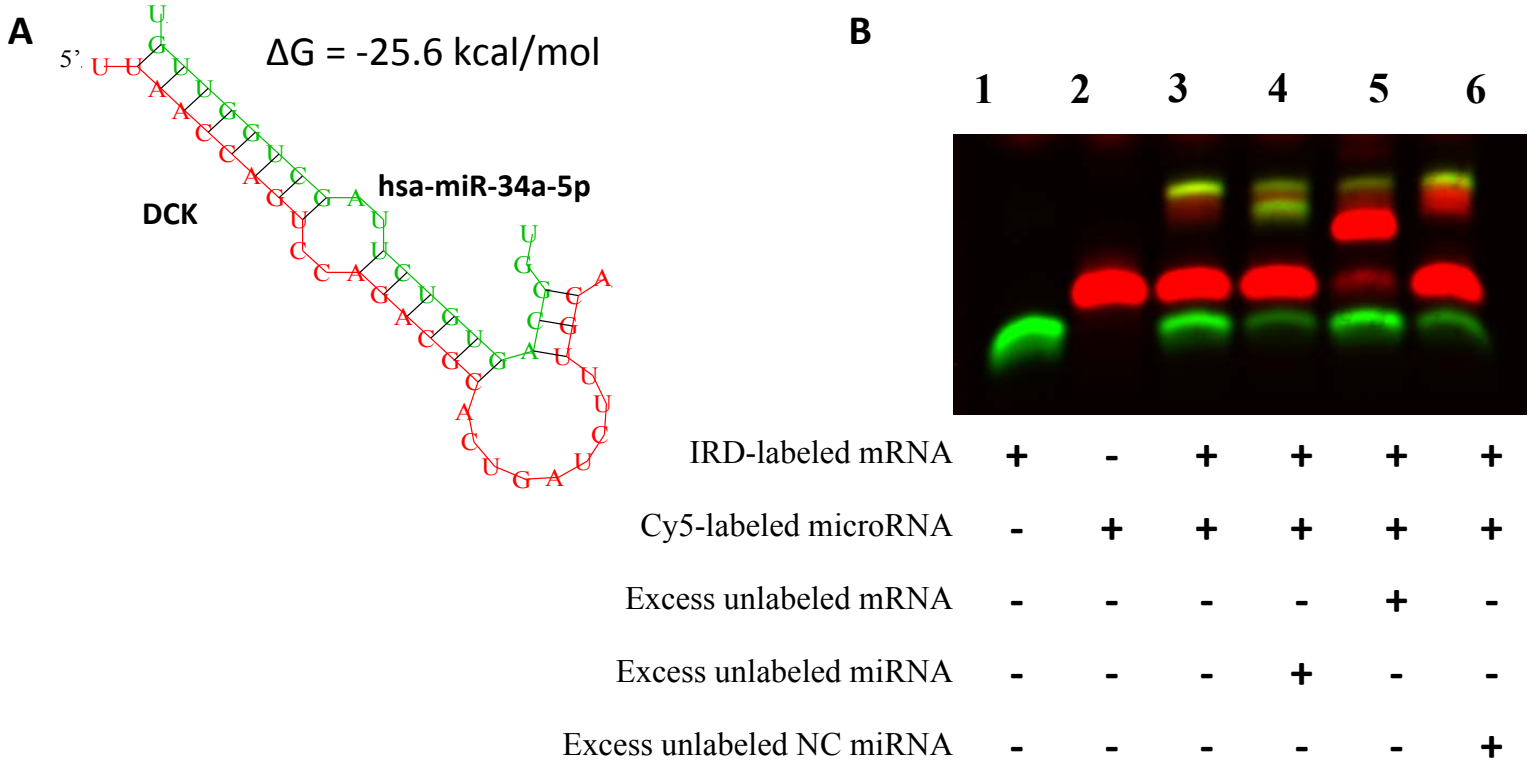
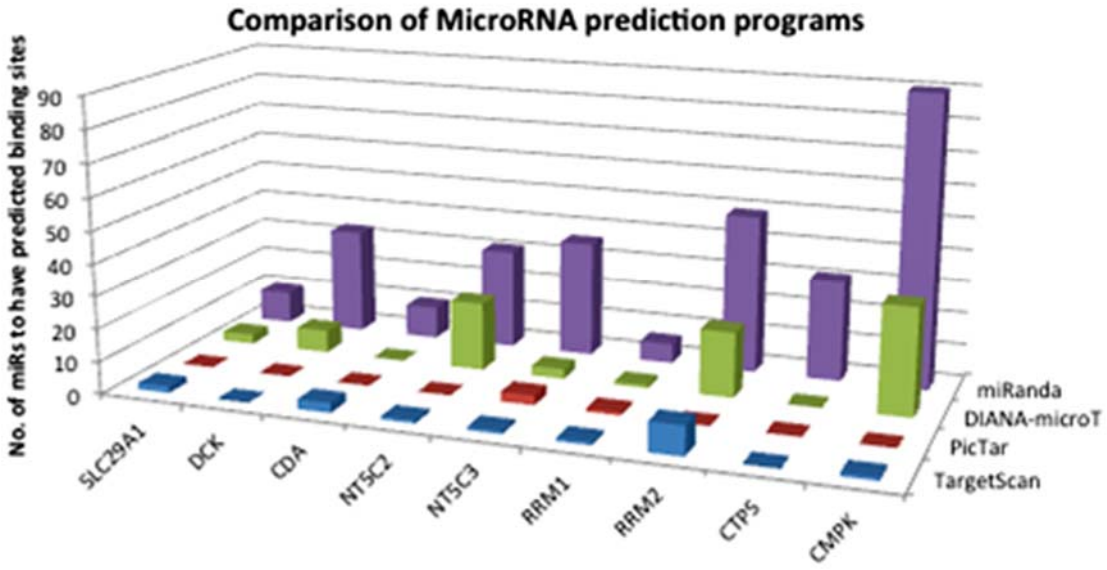


Figure 3.5: Validation of binding interaction between DCK mRNA and hsa-miR-34a-5p by RNA EMSAs. RNA EMSA with cy5-labeled hsa-miR-34a-5p oligonucleotide and 2'-O-methyl modified and IRD-800 labeled DCK mRNA oligonucleotide. Lanes 1 and 2 show the mobility of the labeled mRNA or microRNA oligonucleotide. Lane 3 shows the mobility of the labeled hsa-miR-34a-5p oligonucleotide with DCK mRNA oligonucleotide. Lanes 4 and 6 show the mobility of labeled DCK mRNA oligonucleotide in presence of unlabeled excess specific competitor (hsa-miR-34a-5p) and excess unlabeled non-specific competitor (NC)



Supplementary figure 3.1: Comparison of microRNA prediction programs for predicting binding sites on nucleoside analog pathway genes.

Table 3.1: MicroRNAs significantly associated with nucleoside analog pathway genes in AML Cell lines

Pathway Genes	MicroRNAs	Spearman r	P value
CDA	hsa-miR-23a-3p	0.9524	0.0011
DCTD	hsa-miR-24-3p	-0.9341	0.0011
CTPS	hsa-miR-181a-5p	0.8743	0.0072
DCK	hsa-miR-96-5p	-0.9048	0.0046
DCK	hsa-miR-34a-5p	-0.8810	0.0072
NT5C3	hsa-miR-149-5p	-0.8810	0.0072
RRM2	hsa-let-7g-5p	0.9762	0.0004
RRM2	hsa-miR-151a-3p	-0.9524	0.0011
RRM2	hsa-miR-142-3p	0.9048	0.0046
RRM2B	hsa-miR-194-5p	-0.8810	0.0072
CMPK	hsa-miR-1301	-0.9762	0.0004
CMPK	hsa-miR-320e	-0.9524	0.0011
CMPK	hsa-miR-1323	-0.9286	0.0022
CMPK	hsa-miR-584-5p	-0.9286	0.0022
CMPK	hsa-miR-30d-5p	0.9048	0.0046
CMPK	hsa-miR-381	-0.8810	0.0072
CMPK	hsa-miR-507	-0.8810	0.0072
CMPK	hsa-miR-605	-0.8810	0.0072
CMPK	hsa-miR-762	-0.8810	0.0072
CMPK	hsa-miR-769-3p	-0.8810	0.0072
CMPK	hsa-miR-891a	-0.8810	0.0072
NME1	hsa-miR-514b-5p	-0.9286	0.0022
NME1	hsa-miR-542-3p	-0.9286	0.0022
NME1	hsa-miR-570-3p	-0.9048	0.0046

NME1	hsa-miR-646	-0.9048	0.0046
NME1	hsa-let-7e-5p	0.8810	0.0072
NME1	hsa-miR-224-5p	-0.8810	0.0072
NME1	hsa-miR-761	-0.8810	0.0072
NME1	hsa-miR-767-5p	-0.8810	0.0072
SLC28A1	hsa-miR-548aa	-0.9643	0.0028
SLC28A3	hsa-miR-448	-0.9643	0.0028

Table 3.2: Common MicroRNAs significantly associated with nucleoside analog pathway genes in TCGA database and in vitro study

Gene	Gene Probe ID	MicroRNAs	Spearman r	P value
CDA	205627_at	hsa-mir-23a	0.3731	0.0000
DCTD	201572_x_at	hsa-mir-24-2	-0.2153	0.0032
DCTD	210137_s_at	hsa-mir-24-2	-0.2091	0.0042
CTPS	202613_at	hsa-mir-181a-2	0.2359	0.0012
NT5C3	240128_at	hsa-mir-149	-0.2502	0.0006

Table 3.3: Free Energy Calculation Analysis of the interaction between microRNAs and mRNAs

mRNA + microRNA pair	Minimum Free Energy (mfe) (kcal/mol)
DCTD + hsa-miR-24-3p	-27.2
DCK + hsa-miR-34a-5p	-25.6
DCK + hsa-miR-96-5p	-24.3
NT5C3 + hsa-miR-149-5p	-21.4
CDA + hsa-miR-23a-3p	-19.0

RRM2 + hsa-miR-151a-3p	-26.4
RRM2B + hsa-miR-194-5p	-21.6
CMPK1 + hsa-miR-1301	-23.6
CMPK1 + hsa-miR-320e	-20.1
CMPK1 + hsa-miR-1323	-22.3
CMPK1 + hsa-miR-584-5p	-23.7
CMPK1 + hsa-miR-381	-25.8
CMPK1 + hsa-miR-507	-19.1
CMPK1 + hsa-miR-605	-23.1
CMPK1 + hsa-miR-762	-29.2
CMPK1 + hsa-miR-769-3p	-27.6
CMPK1 + hsa-miR-891a	-22.9
NME1 + hsa-miR-514b-5p	-22.0
NME1 + hsa-miR-542-3p	-19.3
NME1 + hsa-miR-570-3p	-22.2
NME1 + hsa-miR-646	-25.9
NME1 + hsa-miR-224-5p	-17.9
NME1 + hsa-miR-761	-24.3
NME1 + hsa-miR-767-5p	-28.8
SLC28A1 + hsa-miR-548aa	-16.3
SLC28A3 + hsa-miR-448	-23.8

CHAPTER IV

Clinical Significance of *In Vivo* Cytarabine-induced Gene Expression Signature in AML

This chapter has been published in Leukemia and Lymphoma. 2015 Oct 16:1-12

4.1. Abstract

Despite initial remission, ~60–70% of adult and 30% of pediatric patients experience relapse or refractory AML. Studies so far have identified base line gene expression profiles of pathogenic and prognostic significance in AML; however, the extent of change in gene expression post-initiation of treatment has not been investigated. Exposure of leukemic cells to chemotherapeutic agents such as cytarabine, a mainstay of AML chemotherapy, can trigger adaptive response by influencing leukemic cell transcriptome and, hence, development of resistance or refractory disease. It is, however, challenging to perform such a study due to lack of availability of specimens post-drug treatment. The primary objective of this study was to identify in vivo cytarabine-induced changes in leukemia cell transcriptome and to evaluate their impact on clinical outcome. The results highlight genes relevant to cytarabine resistance and support the concept of targeting cytarabine-induced genes as a means of improving response.

4.2. Introduction

In treating AML, refractory disease remains the greatest challenge. Despite initial remission, approximately 60-70% of adult patients and 30% of pediatric patients will die of relapse and refractory AML [1, 2]. Moreover, standard chemotherapy treatment with cytarabine and anthracyclines cause significant adverse events including myelosuppression, high risk for infections, need for transfusions, mucositis, neurotoxicity, and cardiotoxicity [3, 4]. Anti-leukemia drugs such as cytarabine act intra-cellularly by incorporation into DNA. Inefficient cellular uptake, reduced intracellular

activation, increased degradation, or expansion of the dNTP pools could result in development of cellular resistance to cytarabine [5]. Although intracellular cytarabine-triphosphate levels correlate with AML response and small increases in cytarabine-triphosphate result in increased AML cell-cytotoxic effect, prior attempts in AML patients to increase cytarabine dosing did not improve clinical outcomes [6]. Together, these data suggest that cytarabine resistance involves other mechanisms.

Previously, we used gene-expression array to identify diagnostic AML cell gene-expression signatures predictive of *in vitro* and *in vivo* response to chemotherapy [7]. This study demonstrated a validated method of integrating genomic-data at diagnosis with important pharmacologic and clinical outcomes. Based on this experience, we reasoned that examining gene-expression changes in AML cells after exposure to cytarabine would provide additional information regarding intracellular response to treatment. However efforts to study *in vivo* leukemic cell gene expression changes induced by chemotherapy has been relatively limited. This is primarily due to technical difficulty in obtaining samples at adequate time and in enough quantity to study transcriptomic changes. Our study is unique in this aspect as it reports *in vivo* cytarabine induced gene expression levels in leukemic cells obtained at diagnosis and 24 hr post-cytarabine infusion. Additionally, we also report gene-expression changes corresponding to worse outcomes may explain mechanisms of refractory disease and may serve as potential targets for enhancing response to cytarabine.

4.3. Materials and Methods

4.3.1. Study Population: Twenty-four *de novo* AML patients enrolled on AML02 trial (clinicaltrials.gov identifier NCT00136084) with matched specimens available at diagnosis (pre-treatment) and 24 hours after the start of first dose cytarabine were included in this study [6]. Patients with acute promyelocytic leukemia or Down's syndrome were excluded. Patients were randomly assigned to receive induction 1 therapy consisting of high-dose (3 g/m^2, days 1, 3 and 5) or low-dose (100 mg/m^2, days 1-10) cytarabine along with daunorubicin (50mg/m^2, days 2, 4 and 6) and etoposide (days 2-6) by intravenous infusion. Subsequent therapy was adapted based on diagnostic risk features and induction 1 response measured as minimal residual disease (MRD) as assessed by flow cytometry. The details of study design and clinical outcome are described elsewhere [6]. St. Jude Institutional Review Board approved the study, and informed consent was obtained from parents/guardians and consents/assents from the individuals themselves when appropriate.

4.3.2. Specimen Collection and AML Cell Enrichment: Bone marrow was aspirated at diagnosis (pre-treatment) and 24 h after the start of first dose cytarabine. Enrichment for AML cells was performed using Ficoll-Hypaque density-gradient centrifugation, as previously described [8]. If necessary, specimens were further enriched to achieve > 80% blasts by immunomagnetic sorting (Miltenyi Biotech, Bergisch Gladbach, Germany).

4.3.3. Gene Expression Profiling: Gene expression profiling of AML cells was performed using GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Santa

Clara, CA). Details regarding RNA isolation, labeling of cRNA, and scanning of Affymetrix arrays have been published previously [9].

4.3.4. Pharmacology Measurements: AML cells from diagnostic bone marrow specimens were treated in vitro with various concentrations of cytarabine (range, 0.002 – 2.5 ng/μL) followed by MTT assays to determine the median lethal concentration (LC50) value as previously described [7, 9].

4.3.5. Clinical Outcomes: Patients were classified as having low-risk AML if the AML cells harbored t(8;21), inv(16), or t(9;11) chromosome abnormalities. High-risk AML included those with del(7), FLT3-ITD mutation, t(6;9), megakaryoblastic AML, treatment-related AML, or AML arising from MDS. All other patients were classified as standard-risk AML. IWG AML response criteria were used to classify clinical responses [10]. Flow cytometry was used to measure minimal residual disease (MRD at day 22), as previously described [11]. MRD was defined as 1 or more AML cells per 1000 bone marrow mononuclear cells (i.e., ≥ 0.1%). Overall study design, patient characteristics and details of endpoints are provided in Table 4.1 and Supplementary Figure-4.1S shows overall study schema.

4.3.6. Statistical Analysis: For each subject and each probe-set, the expression-change was defined as the log-transformed MAS5.0 normalized signal of the 24-hour sample minus that of the baseline sample so that a positive/negative change indicates that expression increased (decreased) from baseline to post-cytarabine infusion.

An arm-stratified signed-rank test was used to identify probe-sets with significant expression changes for both arms and the rank-sum test was used to identify probe-sets

with expression changes that differed significantly between cytarabine treatment arms. The following statistical testing procedure was performed for each probe-set. The Wilcoxon signed-rank test statistic was computed from the expression changes separately for each arm. We also computed the expected value and variance of these statistics under the null hypothesis that the median expression change in the population equals zero. The final z-statistic was computed by subtracting the sum of the null expectations from the sum of the signed-rank statistics and dividing the result by the square root of the sum of the null variances. A final p-value was computed by comparing the final z-statistic to the standard normal distribution.

For each probe-set, the rank-sum test statistic was computed to test the null hypothesis that the two arms had equal median expression changes. The p-value was determined by comparing the observed statistic to a set of statistics obtained by 10,000 random permutations of the assignment of the arm label to ranks of the expression change values. The expected value and variance of the rank-sum statistics under the null hypothesis were used to z-transform the rank-sum statistic.

PRojection-Onto-the-Most-Interesting-Statistical-Evidence (PROMISE) [12] was used to explore the association of expression changes with LC50, MRD, and EFS. Event-free survival (EFS) was defined as the time elapsed from protocol enrollment to the earliest of disease resistance, relapse, death, development of a second malignancy or death, with times for subjects living and free of these treatment failures censored at last follow-up. For this analysis, MRD was numerically represented as 0 (no detectable disease), 1 (between 0.1% and 1% of cells are leukemic), or 2 (>1% of cells are leukemic). An arm-

stratified Spearman-rank correlation statistic was used to characterize the association of expression changes with LC50 and MRD. The sign of this statistic indicates the direction of association of the expression change with these two endpoints. The statistic of Jung et al. [13] was used to characterize the association of expression changes with the duration of EFS. A positive value of this statistic indicates that an increased expression value associates with longer EFS. For each probe-set, the PROMISE statistic was defined as the sum of the LC50 association statistic and the MRD association statistic minus the EFS association statistic. A positive value of the PROMISE statistic indicates a beneficial pattern of association in the sense that a greater expression change value associates with lower LC50 (greater sensitivity to cytarabine), lower MRD (less residual disease after one course), and a lower rate of EFS treatment failures. A negative value of the PROMISE statistic indicates a detrimental pattern of association in the sense that a greater expression change value associates with greater LC50 (greater resistance to cytarabine), greater MRD (more residual disease after one course), and a greater rate of EFS treatment failures. P-values were determined by 10,000 arm-stratified permutations of the assignment of the entire expression change profile to the vector of endpoint data values. For each analysis described above, the robust FDR method of Pounds and Cheng [14] was applied to the p-values to obtain estimates of the false discovery rate. These estimates are reported as q-values.

4.3.7. *In vitro* validation by siRNA mediated knock down of selected genes:

Fourteen genes identified in PR2 analysis, five from PR3 analysis and five that were significant in both PR2 and PR3 analysis were targeted by a rapid and high-throughput

siRNA-drug modifier screening in THP-1 cell lines. THP-1 cells were transfected with three individual siRNAs per selected gene and was tested alongside standard transfection controls (three replicate experimental plates were utilized). DCK was used as a positive control. Since DCK is a rate-limiting enzyme in activation of cytarabine to cytarabine monophosphate, its knock down should increase drug resistance. Post-siRNA transfection, cells were treated with different concentrations of cytarabine (0μM; 0.1μM-IC10; 0.8μM-IC50; and 10μM-IC90) for 48 hours, followed by multi-parametric nuclear morphometry assays using automated microscopy to document the individual and combined phenotypic effects of siRNA gene silencing and cytarabine on cell growth and proliferation. The following definiteness parameters were used to quantify changes in nuclear morphometry:

i) Number of nuclei, absolute number of nuclei per image field, indication of cell proliferative activity;

ii) Condensed nuclei index (% condensed nuclei), the percentage of nuclei classified as having condensed chromatin (defined by intensity and granularity). Serves as an indication of apoptotic and mitotic nuclei;

iii) Aberrant nuclei index (% deformed "aberrant nuclei), the percentage of nuclei classified as misshapen (defined by circularity and elliptical fit);

iv) Large nuclei index (% large nuclei), based on a size cut-off.

v) Small nuclei index (% small nuclei) based on a size cut-off.

Normalization of all well means was done using corresponding negative controls per cell line, time-point and compound concentration. For controls, median and standard

deviation of three normalized wells per plate, determination of %CV as an expression of intra-plate variation was done. Median and standard deviation of individual normalized wells over three replicate plates and determination of %CV as an expression of inter-plate variation, were in the acceptable range. Normalized siRNA effect on compound-treated cells [normalized well means (IC10; IC50 or IC90) / normalized well means (buffer control)] was determined.

Two methods were utilized to determine siRNA mediated effect.

- *RSA ranking*: The hit selection algorithm, RSA or "redundant siRNA activity", uses an interactive hypergeometric distribution formula to calculate the statistical significance of the siRNA phenotypic readout and ranking for individual genes (indicated by Log P value). By considering the effect of all three siRNA for a gene, and not a single high value siRNA, this algorithm is more sophisticated in its handling of outliers. Because it uses ranks, it does not depend on an underlying data distribution (e.g., Gaussian). RSA analysis using customized scripts (adapted from König et al. [15]) was carried out on normalized siRNA effect on compound-treated cells for all readouts. For maximum flexibility of downstream analysis, RSA was run "in both directions" considering as positives either high or low values (i.e. increase or decrease of phonotypic effect under drug treatment vs. buffer control). Hit selection 1 (RSA analysis): Normalized siRNA effect on compound-treated cells was determined at all inhibitory concentrations levels independently.
- *Cut-off strategy*: A simple way to arrive at a list of hits is to apply a cut-off threshold (2 x SD of negative control) for the number of nuclei (normalized siRNA effect on

compound-treated cells). Two out of three siRNAs per target gene should pass this threshold.

4.4. Results

Among the patients included in this study, 25% were classified as low-risk AML, 42% as standard-risk, and 33% as high-risk. MRD ≥ 0.1 was present in 33% of patients after the first cycle of cytarabine induction chemotherapy.

4.4.1. AML Gene Expression Changes Induced by Cytarabine: An arm-stratified signed-rank test was used to identify probe-sets with significant expression changes for both arms. We identified 51 genes with significant increase and five genes with significant decrease in expression after exposure to cytarabine chemotherapy ($p \leq 0.001$, $q = 0.34$; Table 4.2). Table 4.2 gives the z-statistic, p-value, and robust FDR estimate (q-value) for the probe-sets with $p \leq 0.001$. A positive z-statistic indicates that expression of the probe-set showed a significant increase during the cytarabine infusion and a negative z-statistic indicates that expression of the probe-set showed significant decrease during the cytarabine infusion.

Several genes hold strong potential for biological and clinical relevance. Specifically, we found change in expression of the DNA excision repair genes *DDB2* (1.9-fold increase, $p = 0.0003$,$q = 0.12$) and *ERCC1* (1.4-fold decrease, $p = 0.0006$,$q = 0.34$) after cytarabine. Components of the PI3K/Akt activation pathway, including *AKTIP* showed increased expression after cytarabine ($p = 0.0002$,$q = 0.12$). STAT1 and STAT3, signal transducer and transcription activators involved in multiple pathways (FLT3 signaling, MAPK and Jak/Stat signaling pathways, etc.), were increased post cytarabine treatment ($p = 0.0007$

and 0.001, respectively). Among other genes of significant biological/clinical interest that were increased in expression by cytarabine ($p < 0.001$) included FYN, a member of tyrosine kinase oncogene family, MAX-MYC associated protein is an oncoprotein, CDKN1A (p21CIP1), a cyclin dependent kinase inhibitor; GTPAses-GIMAP4 and GIMAP6, transmembrane receptors (TNFRS10B, TNFRS25, CLEC4A and CD3E) involved in regulating caspases, protein phosphatases (PPP2R2B and DUSP5); transporters (ATP6V1C1 and SLC4A1), transcription regulators (KMT2A, SIX2,TRIP4, ZKSCAN1). Analysis by Ingenuity pathway analysis tool mapped these genes to Tec Kinase, JAK/Stat, ERK/MAPK, Prolactin and Ephrin, IL22 and CTLA4 signaling pathways (Figure 4.1).

4.4.2. Effect of cytarabine dose gene expression changes: To determine whether cytarabine dose impacted changes in gene-expression, patients were categorized into two groups (high dose vs. low dose) according to the cytarabine dose received during first-induction chemotherapy. The two groups did not differ in age, sex, race, or molecular-translocations (Table 4.1). Changes in AML cell gene-expression were not different between the two treatment groups, after consideration of multiple testing ($q = 1.0$; Supplementary Table 4.1S). This result is congruent with the AML02 clinical trial results, which demonstrated no significant differences in day 22 MRD levels or EFS between the two randomized doses of cytarabine [6].

4.4.3. PROMISE Analysis: We then used the PROMISE statistical procedure to identify gene with expression changes that were associated with detrimental outcomes (higher LC50, positive MRD at day 22, and a longer event-free survival time period) OR

beneficial outcomes (lower LC50, negative MRD, shorter EFS). When analyzing for all three outcomes of interest (PR3 analysis), 65 genes were significantly ($p \leq 0.001$, $q=0.32$) associated with beneficial or detrimental outcome. Thirteen of 65 (20%) genes were associated with detrimental response and 52/65 genes were associated with beneficial response ($p < 0.001$, and Figure 4.2A). Because LC50 data was unavailable for some study participants ($n = 16$), we performed PROMISE analysis with two clinical outcomes of interest (MRD and EFS: PR2 analysis). In this analysis, we identified 32 genes as significantly associated with clinical response ($p \leq 0.001$, $q=0.72$, Figure 4.2B). None of identified genes in this study associated with AML risk group assigned at diagnosis ($p > 0.05$ for each gene). Genes with significant association at $q < 0.3$ are summarized in Tables 4.3 and 4.4. Figures 4.3A-D illustrates the association of the expression changes of selected probe-sets (identified in PR2/ PR3 analysis) with MRD and event free survival (EFS). PB1, polybromo1, was the top gene in PR2 analysis with expression change predictive of MRD22 and EFS (PR2, $p = 0.000$; MRD $p = 0.0026$; EFS $p = 0.0005$, Figure 4.3A). PB1 is involved in transcriptional activation and repression of genes involved in chromatin remodeling and acts as a negative regulator of cell proliferation. Change in TRIM33, a transcriptional repressor with a role in cell proliferation, was associated with a favorable outcome (PR3, $p = 0.0001$, $q = 0.117$; EFS $p = 0.0001$; MRD $p = 0.0238$, Figure 4.3B). TRIM33 has been shown to mediate erythroid differentiation of hematopoietic stem/progenitor in response to TGFβ [16]. Similarly MLNR expression change was also predictive of clinical outcome, (PR2, $p = 0.0001$; MRD $p \leq 0.0001$ and EFS $p = 0.02$, Figure 4.3C). Increased expression of

APOBEC2, a cytidine-deaminase family member was associated with a beneficial pattern of association (PR3, $p < 0.0001$, $q = 0$; EFS $p = 0.01$; MRD $p = 0.009$; Figure 4.3D). HLA-DQA1- belonging to HLA class II alpha chain paralogues was associated with unfavorable outcome (PR2, $p = 0.002$; EFS $p = 0.04$ and MRD $p = 0.0004$); haplotypes within this and other members of the HLA family have been implicated in the risk of developing CML and ALL [17, 18]. An increase in the expression of RUNX2 (AML3), a member of the RUNX family, showed a beneficial association pattern (PR2, $p = 0.0006$, $q = 0.51$; MRD $p = 0.0008$). Fusion of the RUNX family gene RUNX1 with ETO is considered a low-risk feature that is associated with a better prognosis [19]. Increased expression of the nuclear oncogene SET showed a detrimental association pattern (PR3, $p = 0.0007$, $q = 0.30$; MRD $p=0.01$). Increased expression of DKK3, a tumor suppressor that inhibits WNT oncogenic signaling and is involved in the regulation of mortalization-related gene expression [20], showed a beneficial association pattern (PR2, $p = 0.0008$; EFS $p = 0.0017$; MRD $p = 0.03$).

4.4.4. *In vitro* validation of selected genes using siRNA mediated knockdown: After identifying inducible genes that were also associated with clinical importance, we next questioned whether targeting these genes could modify leukemia response to cytarabine. Fourteen genes identified in PR2 analysis, five from PR3 analysis and five that were significant in both PR2 and PR3 analysis were targeted by a rapid and high-throughput siRNA-drug modifier screening in THP-1 cell lines (Table 4.5). Each gene was targeted with three individual siRNAs and was tested alongside standard transfection controls (DCK was used as a positive control). Post-siRNA transfection, cells were treated with

different concentrations of cytarabine (0μM; 0.1μM-IC10; 0.8μM-IC50; and 10μM-IC90) followed by multi-parametric nuclear morphometry assay using automated microscopy to document the individual and combined phenotypic effects of siRNA gene silencing and cytarabine on cell growth and proliferation. siRNA mediated knockdown of CHI3L, NFKB2, APOBEC3G, REPIN1, or DOCK6 increased cytarabine-sensitivity; while knockdown of ADRBK1, NPAS3, SCARB1, TIGD6, or TNC, increased cytarabine-resistance (Table 4.5).

4.5. Discussion

In this study we examined cytarabine induced *in vivo* gene expression changes in pediatric AML patients. Most of the studies in literature have focused on gene expression profiling of the of diagnostic chemo naïve tumor specimens. The knowledge gained from gene expression signature identified in diagnostic specimens have opened up opportunities for biomarker identification as well as identification of potential targets of drug development. In our previous work we have used gene expression array to identify an AML cell gene expression signature that predicts intracellular ara-CTP concentration and clinical response to chemotherapy [7]. This study demonstrated a validated method of integrating genomic data at the time of diagnosis with important pharmacologic and clinical outcomes. Based on this experience and the gene expression changes by chemotherapy, we reasoned that examining gene expression changes in AML cells after exposure to cytarabine would provide relevant information regarding intracellular response to treatment. However there is no study to the best of our knowledge that reports

in vivo gene expression changes induced by cytarabine. One of the challenges to perform such a study is technical difficulty in obtaining clinical samples especially post treatment. One of the unique and significant feature of our study was availability of bone marrow samples 24hr post initiation of cytarabine infusion, since no other chemotherapeutic agent was yet initiated, samples obtained at this time point reflect gene expression differences unique to cytarabine. To the best of our knowledge, this is the first study reporting *in vivo* gene-expression changes that occur during cytarabine treatment in pediatric AML patients.

Our analysis of gene expression changes post cytarabine treatment identified genes of biological interest such as components of PI3K/AKT pathway such as AKTIP, genes involved in DNA repair DDB2 and ERCC1. AKTIP regulates protein kinase B (PKB)/Akt signaling (critical for cell growth, glucose-metabolism, and apoptosis) by enhancing the phosphorylation of PKB regulatory sites [14]. This result confirms and extends findings from our previous work that showed significant correlation between diagnostic AML cell gene expression of PIK3C3 (involved in the PI3K/PTEN/Akt/mTOR signaling cascade) and worse clinical outcomes [7].

Identification of genes that were mapped to Tech signaling is of potential interest given that fact that Bruton's Tyrosine Kinase (BTK), a member of Tec kinase family and a key regulator of B-cell Receptor (BCR) is being explored as a potential target in lymphoma and leukemia [21]. Pharmacological screening of ibrutinib as inhibitor of BTK kinase has shown promising results in AML warranting clinical evaluation [22]. Interestingly we did not observed significant impact of cytarabine dose on gene-expression change; this was

consistent with the clinical outcome that demonstrated no significant difference between the two randomized doses of cytarabine.

Additionally, gene expression changes corresponding to worse outcomes may explain mechanisms of refractory disease and may serve as potential targets for enhancing response to cytarabine. The PROMISE method to identify gene expression changes that are predictive of clinically meaningful pattern of association with multiple endpoints identified 65 genes in PR3 (LC50, MRD and EFS) and 32 in PR2 (MRD and EFS) analysis ($p < 0.001$). Some of the genes of potential interest as novel therapeutic agents include: PB1 (aka PBRM1, BAF180), PolyBromo 1 is a bromodomain protein codes for a subunit of ATP-depend chromatin remodeling complex (SWI/SNF-A), cytarabine induced expression of PB1 to be predictive of better outcome ($p < 10^{-4}$). PBRM1 mutations have been found to be frequent in cancer and in renal cell carcinoma approximately 40% of tumor samples have been shown to harbor PBRM1 mutations. Loss of PB1 expression has been associated with poor prognosis and studies in renal carcinoma suggest PBRM1 to be a tumor suppressor by acting as a targeting subunit of nucleosome remodeling complex [23-25]. P53 transcriptional activity has also been shown to be dependent on PBRM1, thereby resulting in onset of cancer with loss of PBRM1 [26-28]. Although PBRM1 has not yet been implicated in pathology or prognosis of AML, our results show it as a potential target. Therapeutic manipulation of PBRM1 is being explored in renal cell carcinoma and if successful might open up opportunities to modify treatment strategies in AML. Another gene belonging to bromo-domain family is TRIM33, a transcriptional repressor with a role in cell proliferation.

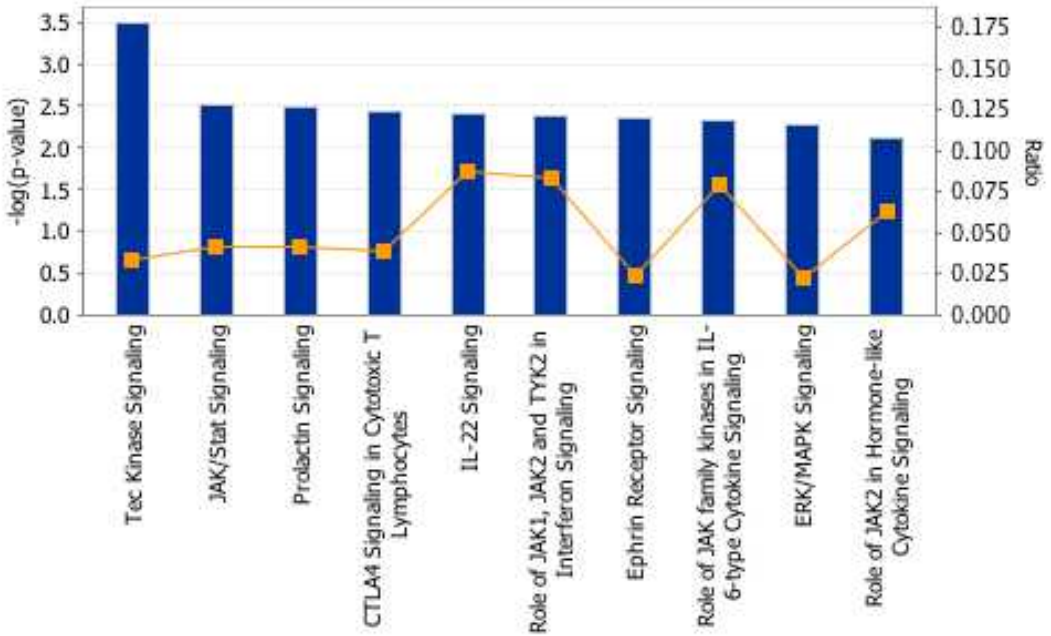
TRIM33 is a multifunctional protein implicated in TGFβ signaling and hematopoietic stem cell (HSC) aging by regulating the balance between lymphoid and myeloid derived HSCs [29]. In mice lacking TRIM33 premature hematopoietic aging has been implicated in predisposition to myeloproliferative disease as CML. TRIM33 has also been shown to act in PARP-dependent DNA damage response by timely removal of ACL1 from damages chromatin, thereby facilitating DNA repair [30]. Our results are in consensus with proposed biological functions of TRIM33 and with supporting evidence form literature opens up potential opportunities to develop therapeutic strategies to module DNA repair efficiency in tumors lacking TRIM33. Among tumor suppressors, another gene of significant clinical value is DKK3, which inhibits WNT oncogenic signaling and is involved in the regulation of mortalization-related gene expression [20]. Down-regulation of DKK3 via promoter hyper-methylation has been associated with poor prognosis in ALL [20], which is concordant with our finding that increased expression of DKK3 associates with beneficial outcomes. Thus, combining a hypomethylating agent such as decitabine with cytarabine might be a strategy to induce expression of DKK3. SET, which is a target of translocation in AML and is involved in tumor metastasis, chromatin remodeling, apoptosis and the MAP/ERK pathway. SET also inhibits the GZMA-activated DNase NME1, a nucleotide diphosphate kinase involved in cytarabine activation [31]. SET antagonism has been implicated in overcoming drug-resistance in myeloid leukemia [32]. This finding is concordant with our result that increased expression of SET is detrimental and suggests JAK2 inhibition as a potential therapeutic strategy for AML.

DIO2, belongs to family of deiodinases that is involved in thyroid metabolism, additionally have been implicated in maintaining balance between proliferation and differentiation. A recent study identified seleno-compounds that can module expression levels of DIO enzymes, thereby allowing modulation of balance between proliferation and differentiation as a therapeutic strategy [33].

Overall our results for the first time report *in vivo* cytarabine induced gene-expression changes in AML. Since cytarabine was the only drug that patients received at that time, the results reflect gene expression changes specific to cytarabine. Drug induced *in vivo* expression changes can often trigger adaptive responses that can contribute to development of resistance or refractory disease. Key genes (such as tumor suppressors DKK3, TRIM33, PBRM1, an oncogene SET, cytidine-deaminase family members APOBEC2 and APOBEC3G) influenced by cytarabine infusion that were also predictive of response can serve as potential targets for enhancing therapeutic strategies. The results highlight genes relevant to cytarabine resistance and support the concept of targeting genes modulated by cytarabine exposure as a means of improving response.

Future in depth studies will help in understanding the interplay of these genes/pathways to better understand mechanisms of cytarabine resistance in AML. Importantly, novel agents directed at these targets may serve as potential therapeutics to improve clinical outcomes. In summary, our results identified genes of potential biological and therapeutic significance that are influenced by cytarabine treatment, thereby opening up opportunities for future research to elucidate the mechanisms underlying AML response/resistance and identify targets of development of novel agents.

(A)


-log(p-value)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ratio
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
Tec Kinase Signaling
JAK/Stat Signaling
Prolactin Signaling
CTLA4 Signaling in Cytotoxic T Lymphocytes
IL-22 Signaling
Role of JAK1, JAK2 and TYK2 in Interferon Signaling
Ephrin Receptor Signaling
Role of JAK family kinases in IL-6-type Cytokine Signaling
ERK/MAPK Signaling
Role of JAK2 in Hormone-like Cytokine Signaling

(B)

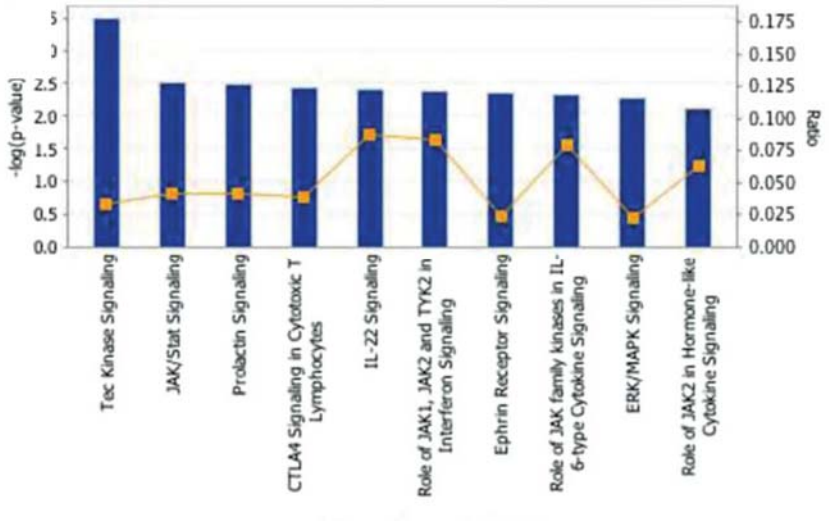
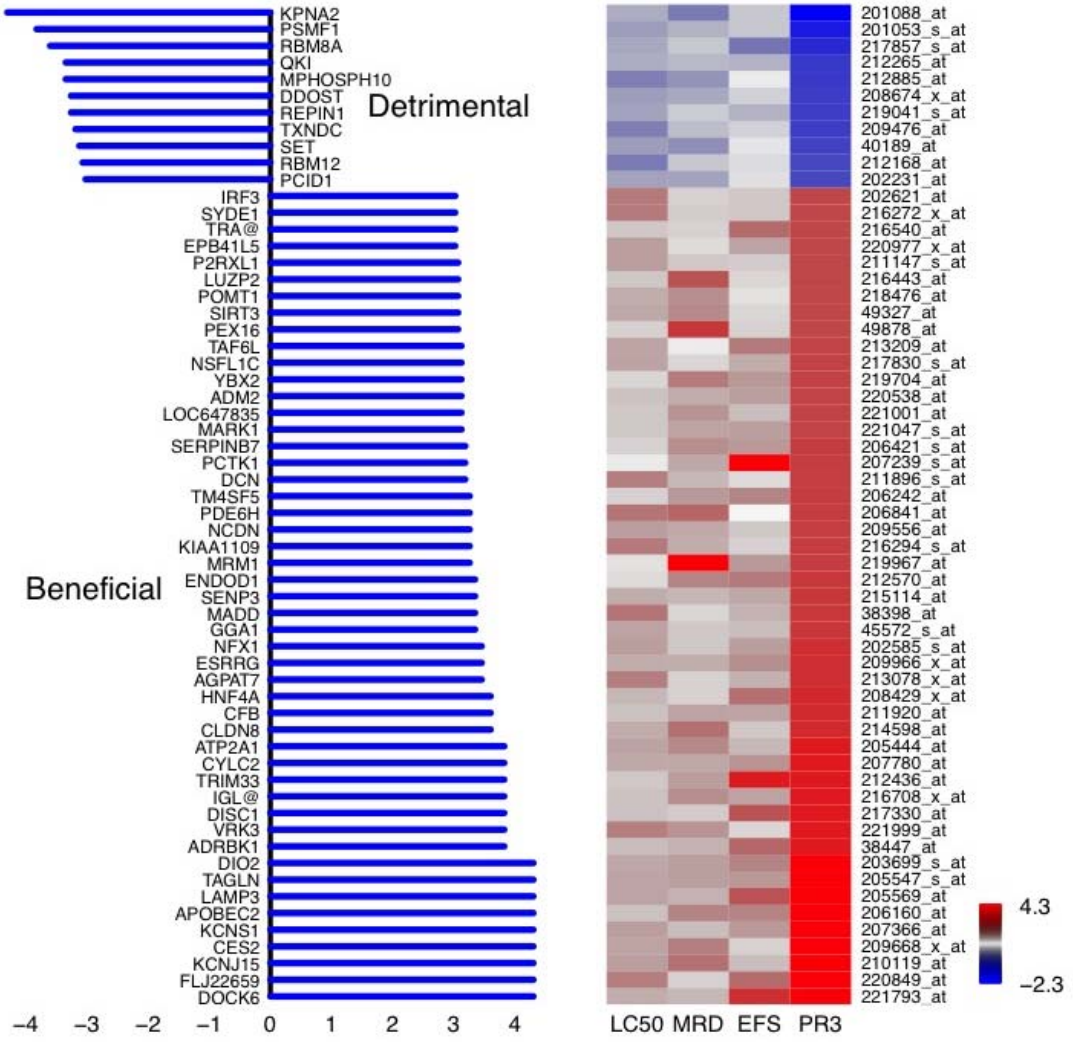


Figure 4.1. Pathway analysis utilizing Ingenuity pathway analysis tool of genes demonstrating significant change in expression post-cytarabine infusion in AML patients. (A) Top 10 canonical pathways for genes with significant change in expression post cytarabine infusion. Y-axis indicates Log p value (calculated with the right-tailed Fisher's Exact Test) and Ratio (percentage of genes in a pathway that were also found in results). The ratio is, therefore, good for looking at which pathway has been affected the most based on the percentage of genes uploaded into IPA. (B) The network of these 10 pathways demonstrating interactions between the pathways due to shared genes.

(A) PR3 Analysis



(B) PR2 Analysis

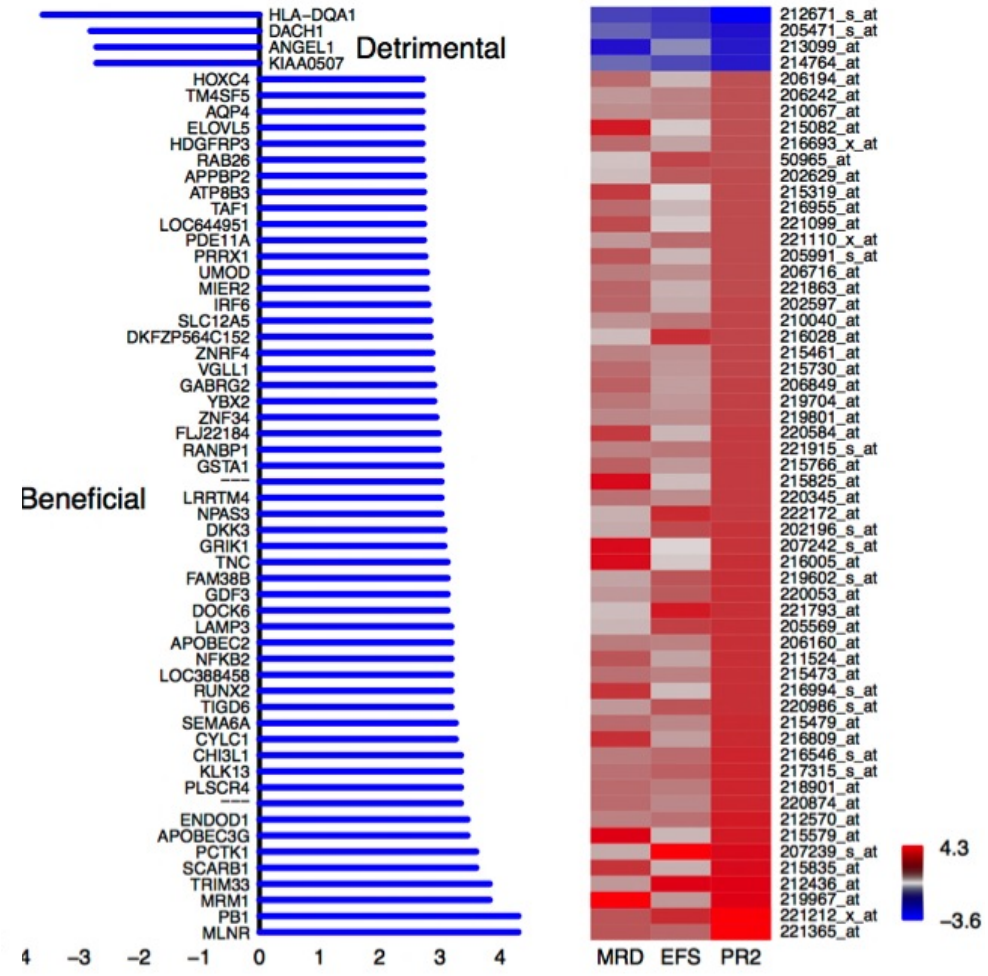
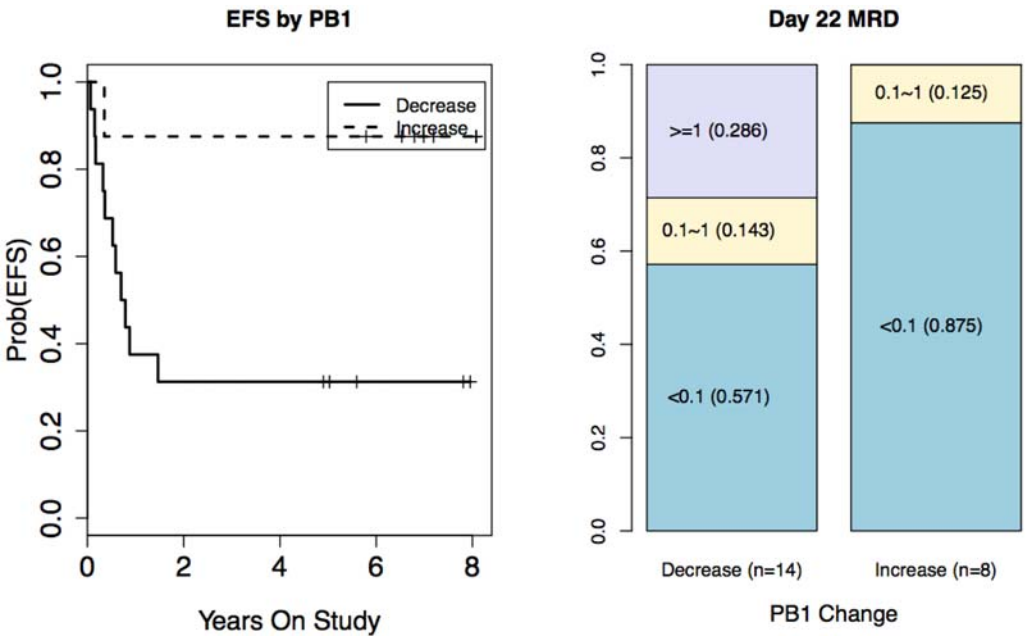


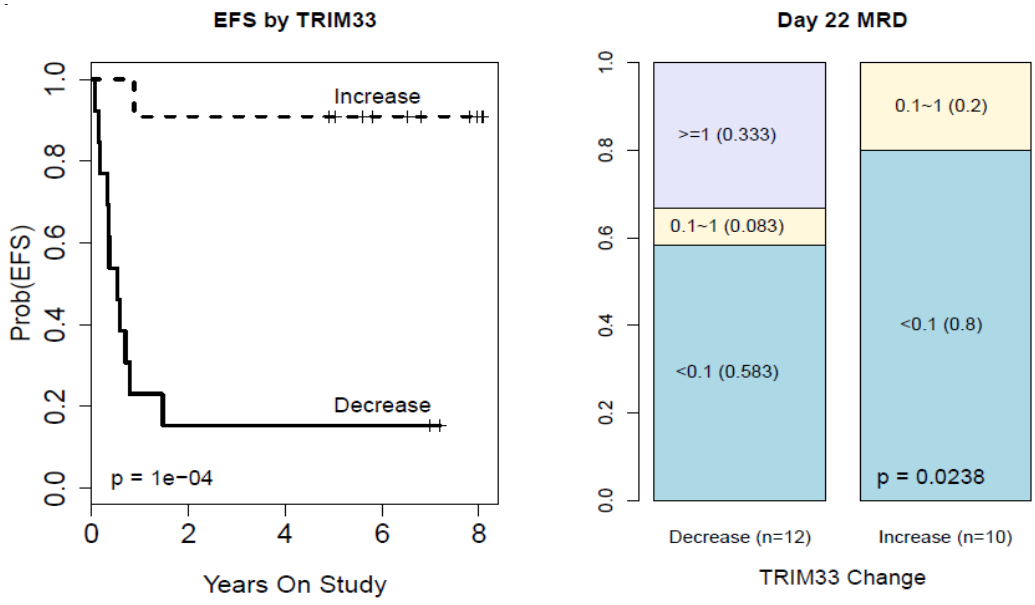
Figure 4. 2.Therapeutically beneficial and detrimental patterns of association detected by the PROMISE method. (A) The three-endpoint PROMISE analysis (PR3) identified 65 probe sets with cytarabine induced changes in expression levels that showed a beneficial or detrimental pattern of association with *in vitro* LC50, MRD and EFS. (B) The two-endpoint PROMISE analysis (PR2) identified 33 probe sets with cytarabine induced change in expression levels that showed beneficial or detrimental patterns of association with MRD and EFS. X-axis values give a log10 p-value with sign defined by

the pattern (negative for detrimental and positive for beneficial). Each row represents a gene and each column represents a clinical endpoint: MRD and EFS; PR2 indicates the statistical values corresponding to the PROMISE analysis. Colors are assigned according to the signed log10 p-value. EFS: Event-free survival; MRD: Minimal residual disease; PROMISE: Projection onto the Most Interesting Statistical Evidence.

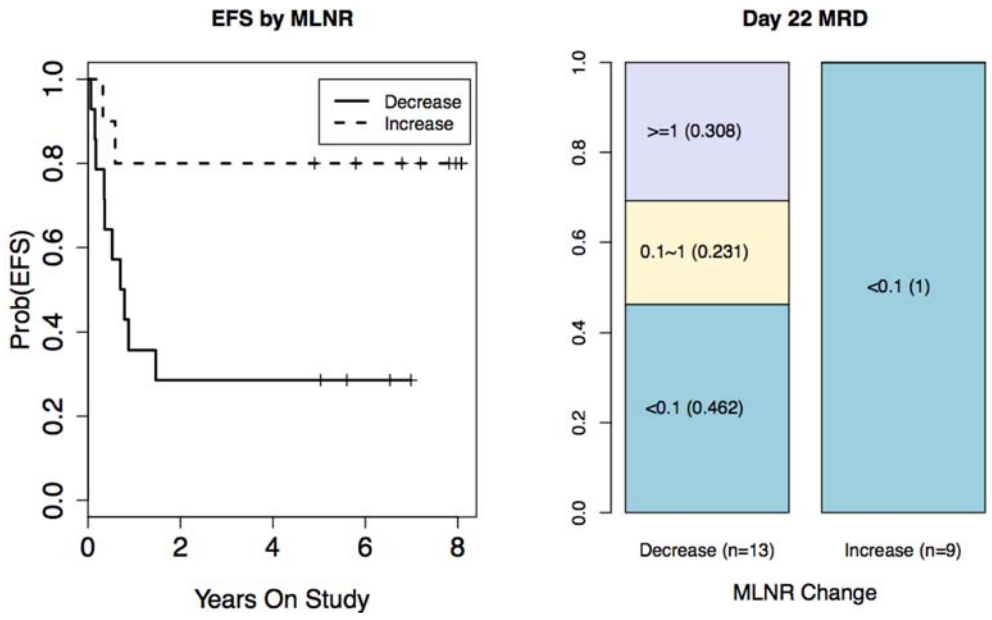
(A)


EFS by PB1
Decrease
Increase
Prob(EFS)
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
Years On Study
Day 22 MRD
>=1 (0.286)
0.1~1 (0.143)
<0.1 (0.571)
0.1~1 (0.125)
<0.1 (0.875)
Decrease (n=14)
Increase (n=8)
PB1 Change

(B)


EFS by TRIM33
Increase
Decrease
p = 1e−04
Prob(EFS)
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
Years On Study
Day 22 MRD
>=1 (0.333)
0.1~1 (0.083)
<0.1 (0.583)
0.1~1 (0.2)
<0.1 (0.8)
p = 0.0238
Decrease (n=12)
Increase (n=10)
TRIM33 Change

(C)



(D)

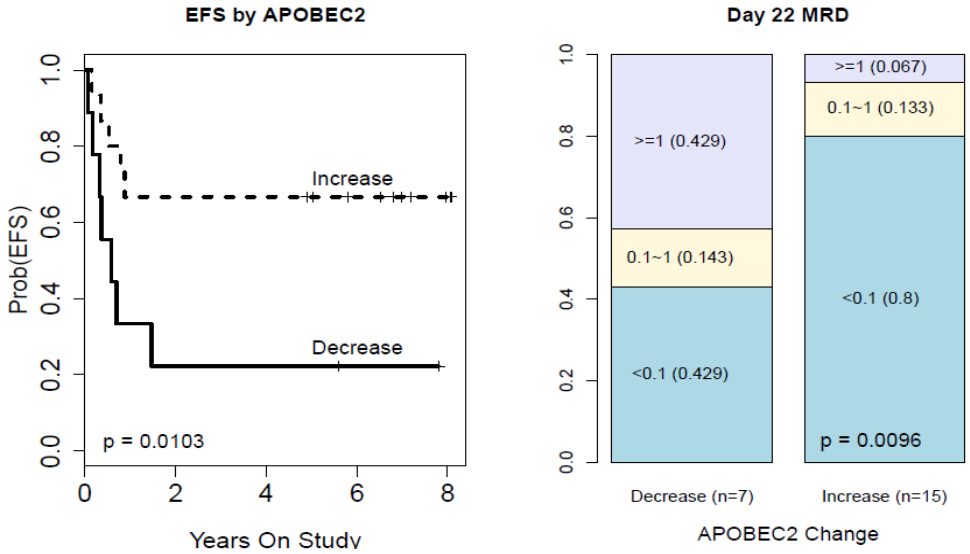
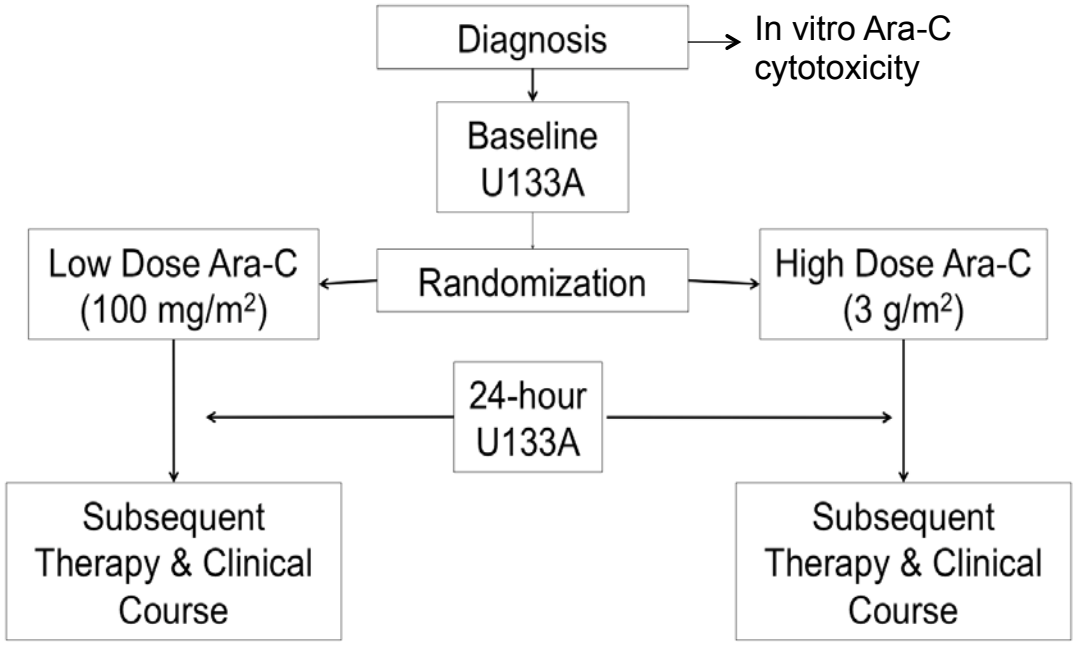


Figure 4.3. Association of cytarabine induced change in expression levels of (A) PB1, (B) TRIM33, (C) MLNR and (D) APOBEC2 with EFS and MRD (day 22) in AML patients.



Supplementary Figure 4.1S: Schematic showing overall study design.

Table 4.1: Patient characteristics by arm

Feature	HDAC	LDAC	P value
Gender			0.089
Female	7	5	
Male	2	10	
Age			0.089
<10 Years	2	10	
>=10 Years	7	5	
WBC			0.657
<50	7	9	
>=50	2	6	
Race			1
Black	1	2	
Other	1	2	
White	7	11	
Cytogenetics			0.277
11q23	4	2	
CBF	3	3	
Normal	1	5	
Other	1	5	
Provisional Risk			0.666
High	2	6	
Low	3	3	
Standard	4	6	

Table 4.2: List of genes with significant change in expression post ara-C infusion in AML patients.

Affy probe ID	Gene Symbol	Gene Name	Statistic	p-value	BH95_q	PC06_q
202284_s_at	CDKN1A	cyclin-dependent kinase inhibitor 1A [p21, Cip1]	165	0.0000	0.1295	0.1203
202665_s_at	WASPIP	HIV-1 Rev binding protein	154	0.0001	0.3179	0.1203
209294_x_at	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	153	0.0001	0.3179	0.1203
213316_at	KIAA1462		152	0.0001	0.3179	0.1203
214054_at	DOK2	docking protein 2, 56kDa	151	0.0001	0.3179	0.1203
202872_at	ATP6V1C1	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C1	150	0.0002	0.3179	0.1203
210105_s_at	FYN	FYN oncogene related to SRC, FGR, YES	150	0.0002	0.3179	0.1203
212357_at	KIAA0280		150	0.0002	0.3179	0.1203
201297_s_at	MOBK1B	MOB1, Mps One Binder kinase activator-like 1B [3][3][2]	150	0.0002	0.3179	0.1203
221653_x_at	APOL2	apolipoprotein L, 2	149	0.0002	0.3179	0.1203
218373_at	AKTIP	AKT Interacting Protein	149	0.0002	0.3179	0.1203
205643_s_at	PPP2R2B	protein phosphatase 2, regulatory subunit B, beta isoform	149	0.0002	0.3179	0.1203
212932_at	RAB3GAP1	RAB3 GTPase activating protein subunit 1 [3][3][2]	149	0.0002	0.3179	0.1203
203217_s_at	ST3GAL5	ST3 beta-galactoside alpha-2,3-sialyltransferase 5	149	0.0002	0.3179	0.1203
203409_at	DDB2	damage-specific DNA binding protein 2, 48kDa	148	0.0003	0.3179	0.1203
202270_at	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	148	0.0003	0.3179	0.1203
215350_at	SYNE1	spectrin repeat containing, nuclear envelope 1	148	0.0003	0.3179	0.1203
214900_at	ZKSCAN1	zinc finger with KRAB and SCAN domains 1	148	0.0003	0.3179	0.1203
219777_at	GIMAP6	GTPase, IMAP family member 6	146	0.0004	0.3682	0.2653
211977_at	GPR107	G protein-coupled receptor 107	146	0.0004	0.3682	0.2653
206765_at	KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2	146	0.0004	0.3682	0.2653
216069_at	PRMT2	protein arginine methyltransferase 2	146	0.0004	0.3682	0.2653
214006_s_at	GGCX	gamma-glutamyl carboxylase	145	0.0005	0.3682	0.2834
219243_at	GIMAP4	GTPase, IMAP family member 4	145	0.0005	0.3682	0.2834

215255_at	IGSF9B	immunoglobulin superfamily, member 9B	145	0.0005	0.3682	0.2834
216841_s_at	SOD2	superoxide dismutase 2, mitochondrial	145	0.0005	0.3682	0.2834
217818_s_at	ARPC4	actin related protein 2/3 complex, subunit 4, 20kDa	144	0.0005	0.3682	0.3324
221042_s_at	CLMN	calmin [calponin-like, transmembrane]	144	0.0005	0.3682	0.3324
209457_at	DUSP5	dual specificity phosphatase 5	144	0.0005	0.3682	0.3324
216080_s_at	FADS3	fatty acid desaturase 3	144	0.0005	0.3682	0.3324
218092_s_at	HRB	HIV-1 Rev binding protein	144	0.0005	0.3682	0.3324
210734_x_at	MAX	MYC associated factor X	144	0.0005	0.3682	0.3324
205456_at	CD3E	CD3e molecule, epsilon [CD3-TCR complex]	143	0.0007	0.3682	0.3393
214551_s_at	CD7	CD7 molecule	143	0.0007	0.3682	0.3393
220546_at	FLJ11783		143	0.0007	0.3682	0.3393
209969_s_at	STAT1	signal transducer and activator of transcription 1, 91kDa	143	0.0007	0.3682	0.3393
202748_at	GBP2	guanylate binding protein 2, interferon-inducible	142	0.0008	0.3682	0.3419
202595_s_at	LEPROTL1	leptin receptor overlapping transcript-like 1	142	0.0008	0.3682	0.3419
207705_s_at	RP4-691N24.1		142	0.0008	0.3682	0.3419
208540_x_at	S100A11	S100 calcium binding protein A11 pseudogene	142	0.0008	0.3682	0.3419
214838_at	SFT2D2	SFT2 domain containing 2	142	0.0008	0.3682	0.3419
206511_s_at	SIX2	SIX homeobox 2	142	0.0008	0.3682	0.3419
219423_x_at	TNFRSF25	tumor necrosis factor receptor superfamily, member 25	142	0.0008	0.3682	0.3419
205299_s_at	BTN2A2	butyrophilin, subfamily 2, member A2	141	0.0010	0.3682	0.3424
221724_s_at	CLEC4A	C-type lectin domain family 4, member A	141	0.0010	0.3682	0.3424
210968_s_at	RTN4	reticulon 4	141	0.0010	0.3682	0.3424
210569_s_at	SIGLEC9	sialic acid binding Ig-like lectin 9	141	0.0010	0.3682	0.3424
208992_s_at	STAT3	signal transducer and activator of transcription 3	141	0.0010	0.3682	0.3424
203567_s_at	TRIM38	tripartite motif-containing 38	141	0.0010	0.3682	0.3424
203732_at	TRIP4	thyroid hormone receptor interactor 4	141	0.0010	0.3682	0.3424
211422_at	TRPM3	transient receptor potential cation channel, subfamily M, member 3	141	0.0010	0.3682	0.3424

221747_at*	TNS1	tensin 1	4	0.0002	0.3179	0.1203
203720_s_at*	ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1	9	0.0005	0.3682	0.3234
205592_at*	IL8	interleukin 8	9	0.0005	0.3682	0.3234
218225_at*	ECSIT	ECSIT homolog [Drosophila]	10	0.0007	0.3682	0.3348
221932_s_at*	GLRX5	glutaredoxin 5	10	0.0007	0.3682	0.3348

Note:

The expected rank (statistic) is 76.5, a statistic greater than 76.5 indicates that there are more positive values in expression change (higher median expression at 24 hours).

*Probes with decrease in expression post-cytarabine.

Table 4.3: PROMISE analysis identified genes change in expression post-cytarabine to be predictive of beneficial or detrimental patterns of association with clinical endpoints in AML patients (q < 0.3). PROMISE 3 (PR3) analysis utilizing three end-points; LC50, MRD22 and EFS

Probe ID	Gene Symbol	LC50 stat	MRD22 stat	EFS stat	PROMISE stat	LC50 p value	MRD22 p value	EFS p value	PR3 p value	BH95_q	PC06_q	Risk p value
201088_at	KPNA2	0.708	0.568	0.290	-0.522	0.044	0.006	0.136	0.000	0.000	0.000	0.171
203699_s_at	DIO2	-0.708	-0.468	-0.506	0.561	0.040	0.026	0.009	0.000	0.000	0.000	0.472
205547_s_at	TAGLN	-0.773	-0.462	-0.447	0.561	0.030	0.026	0.020	0.000	0.000	0.000	0.662
205569_at	LAMP3	-0.773	-0.406	-0.614	0.598	0.028	0.060	0.001	0.000	0.000	0.000	0.686
206160_at	APOBEC2	-0.602	-0.531	-0.493	0.542	0.110	0.010	0.010	0.000	0.000	0.000	0.245
207366_at	KCNS1	-0.773	-0.365	-0.448	0.529	0.024	0.085	0.020	0.000	0.000	0.000	0.879
209668_x_at	CES2	-0.773	-0.537	-0.253	0.521	0.030	0.008	0.203	0.000	0.000	0.000	0.658
210119_at	KCNJ15	-0.773	-0.586	-0.331	0.564	0.027	0.004	0.090	0.000	0.000	0.000	0.531
220849_at	FLJ22659	-0.838	-0.291	-0.549	0.559	0.007	0.177	0.004	0.000	0.000	0.000	0.719
221793_at	DOCK6	-0.708	-0.400	-0.650	0.586	0.043	0.064	0.000	0.000	0.000	0.000	0.886
201053_s_at	PSMF1	0.773	0.403	0.306	-0.494	0.027	0.057	0.116	0.000	0.153	0.118	0.842
205444_at	ATP2A1	-0.773	-0.523	-0.349	0.548	0.029	0.011	0.071	0.000	0.153	0.118	0.259
207780_at	CYLC2	-0.708	-0.435	-0.460	0.534	0.042	0.041	0.017	0.000	0.153	0.118	0.858
212436_at	TRIM33	-0.602	-0.478	-0.668	0.583	0.116	0.024	0.000	0.000	0.153	0.118	0.983
216708_x_at	IGL2	-0.643	-0.520	-0.411	0.525	0.096	0.013	0.033	0.000	0.153	0.118	0.638
217330_at	DISC1	-0.643	-0.307	-0.607	0.519	0.097	0.152	0.001	0.000	0.153	0.118	0.284
221999_at	VRK3	-0.838	-0.504	-0.233	0.525	0.008	0.017	0.246	0.000	0.153	0.118	0.388
38447_at	ADRBK1	-0.643	-0.400	-0.546	0.530	0.091	0.059	0.003	0.000	0.153	0.118	0.809
208429_x_at	HNF4A	-0.659	-0.277	-0.549	0.495	0.066	0.200	0.004	0.000	0.217	0.192	0.854
211920_at	CFB	-0.643	-0.450	-0.408	0.500	0.097	0.031	0.032	0.000	0.217	0.192	0.943
214598_at	CLDN8	-0.708	-0.579	-0.309	0.532	0.043	0.004	0.114	0.000	0.217	0.192	0.435

217857_s_at	RBM8A	0.708	0.325	0.530	-0.521	0.042	0.133	0.005	0.000	0.217	0.192	0.476
202585_s_at	NFX1	-0.773	-0.326	-0.427	0.509	0.027	0.132	0.028	0.000	0.278	0.254	0.941
209966_x_at	ESRRG	-0.667	-0.417	-0.472	0.519	0.050	0.049	0.013	0.000	0.278	0.254	0.355
213078_x_at	AGPAT7	-0.838	-0.291	-0.372	0.500	0.008	0.172	0.052	0.000	0.278	0.254	0.498

Table 4.4: PROMISE analysis identified genes change in expression post-cytarabine to be predictive of beneficial or detrimental patterns of association with clinical endpoints in AML patients ($q < 0.3$). PROMISE 2 (PR2) analysis utilizing two end-points; MRD22 and EFS

Probe ID	Gene Symbol	MRD22 stat	EFS stat	PROMISE stat	MRD22 p value	EFS p value	PR2 p value	BH95_q	PC06_q	Risk p value
221212_x_at	PB1	-0.613	-0.635	0.624	0.003	0.001	0.000	0.000	0.000	0.212
221365_at	MLNR	-0.611	-0.529	0.570	0.002	0.004	0.000	0.000	0.000	0.284

Table 4.5: Effect of siRNA mediated knockdown of selected genes on cytarabine sensitivity in THP1 cells.

Gene symbol	PROMISE analysis	siRNA-drug modifier GENE LEVEL effect (RSA ranking based)	siRNA-drug modifier GENE LEVEL effect (Cut off strategy based)	Non-neutral genes*	Only one of three siRNA influence drug response
ADRBK1	PR3	Increased resistance	Increased resistance		
APOBEC2	PR2 and PR3				Increased sensitivity
APOBEC3G	PR2 and PR3	Increased sensitivity		non-neutral	
CHI3L1	PR2	Increased sensitivity			
CYLC1	PR2				Increased resistance
DCK	+ve control	Increased resistance	Increased resistance		
DOCK6	PR2 and PR3	Increased sensitivity			
ENDOD1	PR2 and PR3				Increased resistance
GDF3	PR2				
GSTA1	PR2				Increased resistance
KLK13	PR2				
LRRTM4	PR2				Increased resistance
MARK1	PR3				
MLNR	PR2				
NFKB2	PR2	Increased sensitivity			
NPAS3	PR2	Increased resistance		non-neutral	Increased resistance
QKI	PR3				
RANBP1	PR2				
REPIN1	PR3	Increased sensitivity	Increased sensitivity		

RUNX2	PR2				Increased resistance
SCARB1	PR2	Increased resistance	Increased resistance		
SET	PR3			non-neutral	
TIGD6	PR2	Increased resistance	Increased resistance		
TNC	PR2	Increased resistance			
TRIM33	PR2 and PR3			non-neutral	

*Note: Non-neutral genes refer to genes, which do yield a detectable RNAi phenotype in the absence of the drug (phenotype was sometimes further enhanced post-drug treatment)

Supplementary Table 4.1S: List of genes with distinct pattern of expression differences post-cytarabine between LDAC and HDAC arms ($p < 0.001$).

Probe Id	Gene symbol	Gene Name	Stat	p-value	q-value
220443_s_at	VAX2	ventral anterior homeobox 2	3.369	0.0006	1
210139_s_at	PMP22	peripheral myelin protein 22	3.2498	0.0003	1
200730_s_at	PTP4A1	protein tyrosine phosphatase type IVA, member 1	3.1901	0.0008	1
201148_s_at	TIMP3	TIMP metallopeptidase inhibitor 3	3.0709	0.0009	1
203241_at	UVRAG	UV radiation resistance associated gene	-3.1901	0.0004	1
221376_at	FGF17	fibroblast growth factor 17	-3.1901	0.0006	1
216698_x_at	OR7E47P	olfactory receptor, family 7, subfamily E, member 47 pseudo gene	-3.3094	0.0006	1
221378_at	CER1	cerberus 1, cysteine knot superfamily, homolog (Xenopus laevis)	-3.4883	0.0004	1

Note: A positive z-statistic indicates that arm A had a greater median expression change than arm

CHAPTER IV [B]

Drug-Induced Gene Expression and MicroRNA Expression Changes in AML Cell lines

4.6. Introduction

Nucleoside analogs are a group of chemotherapeutic drugs, which mimic the endogenous nucleotide structurally as well as functionally. Cytarabine, a deoxycytidine analog, is the cornerstone of the chemotherapeutic regimens for the treatment of acute myeloid leukemia (AML). AML is a very aggressive disease and despite initial remission of the disease, the survival rate is very poor, with almost 60% to 70% of adult AML patients and 30% of pediatric AML patients die of relapse and/or refractory AML [1, 2]. Insufficient cellular uptake of the drug, decreased intracellular activation to the active metabolite, increased inactivation or increase of the endogenous deoxynucleotide triphosphate pools, which compete with the drug for incorporation in growing DNA strand, are potential mechanisms that can result in development of resistance to nucleoside analogs, like cytarabine [3]. *De novo* and acquired resistance to nucleoside analogs limit the effectiveness of therapy, and an effort to understand these mechanisms are under study. Evidence suggests that AML patients who have increased formation and retention of cytarabine triphosphate in leukemic cells experienced increased duration of remission after receiving cytarabine-based remission induction and maintenance therapy [4, 5]. Attempts to introduce higher doses of cytarabine, did not significantly improve the treatment outcomes [6, 7]. Thus, development of resistance to cytarabine could involve additional complex mechanisms. Evidence suggests that prolonged *in vitro* and *in vivo* treatment with cytarabine results in emergence of drug resistance, leading to decreased sensitivity and ultimately treatment failure [4, 8-11]. Also, selection of drug-resistant leukemic cell population may be associated with changes in malignant properties such as

proliferation rate, invasiveness, and immunogenicity. Evidence also indicates that chemotherapy with cytarabine and anthracycline results in modulation of wide range of proteins including p53 protein activation followed by cell cycle arrest and apoptosis [12-14].

Current search in improving treatment outcomes in AML are primarily focused on understanding the factors intrinsic to the leukemic blasts that help predict the outcome in patients and their response to the chemotherapy. Studies have determined the specific cytogenetic and molecular features, such as core-binding factor mutations, inv (16) and t (18; 21) predicting favorable outcome in AML, whereas monsomy 7, del (5q), MLL rearrangements and FLT3-ITD mutations to be associated with poor AML prognosis [15, 16]. Studies have also focused on identifying the gene expression signatures in acute myeloid leukemia, in order to better understand the genes with aberrant expression in AML blast cells, to identify genes associated with AML prognosis and potential therapeutic targets [17]. Multiple independent gene expression-profiling studies in AML identified specific gene expression clusters in AML predicting risk stratification along with predicting significantly different survival rates [18-20], demonstrating the significance of gene-expression profiling in predicting outcomes AML. Gene expression profiles in AML blast cells at diagnosis in were evaluated to identify patterns of association with various clinical and pharmacological factors of therapeutic importance in AML. This study identified genes involved in PI3K/PTEN/AkT/mTOR signaling pathway, G-protein-coupled receptor signaling and leukemogenesis to be displaying detrimental pattern of association [21]. Another study also able to identify gene

expression signature from among the differentially expressed genes between responders and non-responders to the first induction therapy, which predicted treatment response in these patients [22]. Interestingly, AML disease relapse-specific gene expression signature was observed in patients experiencing relapse after high dose cytarabine-based regimens compared to untreated AML patients, identifying specific genes and associated pathways in contributing to mechanism of recurrent disease [23]. Hence, there is sufficient evidence to justify the significance of studying gene expression signatures in AML patients in order to better understand the molecular mechanisms of involved in cytarabine resistance, in order to improve therapy and possibly reverse drug resistance.

Gene expression signatures, apart from predicting response to chemotherapy, could also help predict information regarding intracellular responses to chemotherapeutic agent. Exposure of leukemic cells to cytarabine, which is an antimetabolite, would trigger adaptive responses by influencing the cell transcriptomic profile. Understanding these chemotherapy-induced gene expression changes might provide additional information that might help explain development of acquired resistance to nucleoside analogs like cytarabine and hence explain development refractory or relapsed AML. Studies in breast cancer patients treated with tamoxifen have identified dynamic gene expression changes after early/transient response to tamoxifen treatment, with decreased expression of genes involved in cell cycle and proliferation, while continuous/ late response to tamoxifen resulted in change in expression of estrogen response genes [24]. In addition, gene expression studies in ovarian cancer xenograft models after treatment with carboplatin and carboplatin plus paclitaxel, demonstrated distinct transcriptional response following

drug treatment, which upregulation of genes involved in DNA repair, cell cycle and apoptosis while oxygen-consuming metabolic pathway and apoptosis control genes showed decreased expression [25]. However, there is limited knowledge regarding the changes in the gene expression in AML cells after exposure to cytarabine. Understanding chemotherapy-induced changes in gene expression could potentially have an impact on the prognosis of AML as well as help in understanding the mechanisms of acquired resistance to cytarabine-based regimens.

Interestingly, recent evidence also indicated changes in expression of various microRNAs in different types of cancers after exposure to cytotoxic stimuli such chemotherapy and radiotherapy [26]. Chemotherapy with cisplatin and 5-fluorouracil in esophageal cancer was shown to affect the expression of various microRNAs involved in important biological networks critical for response to cytotoxic agents [27]. Similarly, rifampin treatment in human hepatocytes was demonstrated to both induce and repress the expression of hepatic microRNAs, suggesting that rifampin-mediated regulation of drug metabolizing enzymes may, in part, be due to altered microRNA expression after rifampin treatment [28].

Since changes in microRNA expression have been shown to be dependent on the type of cells as well as the chemotherapeutic drug [29], it is essential to understand the role of cytarabine chemotherapy on the expression of microRNAs in leukemic cells. To our knowledge, there have been no studies till date investigating the impact of cytarabine-treatment on changes in gene expression and microRNA expression. In the first section of this study, we report the effect of cytarabine treatment at two different concentrations on

the changes in microRNA expression in AML cell lines. We also investigated the effect of cytarabine treatment on the expression of pathway genes, in order to understand if the drug treatment itself can have an impact on its activation or inactivation pathway. Also, since it is difficult to obtain matched bone marrow biopsy samples at baseline and post cytarabine treatment from AML patients, not many studies have reported the *in vivo* changes in gene expression post cytarabine infusion. We have also reported (Chapter IVA) the effect of *in vivo* cytarabine treatment on the global transcriptome expression changes in AML patients. Such information could potentially provide important information regarding intracellular responses to treatment as well as development of acquired resistance to cytarabine-based regimens.

4.7. Materials and Methods

4.7.1. Cell Culture and Reagents: The AML cell lines HL-60, MV-4-11, Kasumi-1, THP-1, AML-193 and KG-1 cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA), while MOLM-16 and ME-1 cell lines were obtained from DSMZ (Braunschweig, Germany). Kasumi-1, ME-1 and MOLM-16 cell lines were cultured in RMPI-1640 medium supplemented with 20% fetal bovine serum (FBS), THP-1 cell line was cultured in RPMI-1640 medium supplemented with 10% FBS, MV-4-11 cell line was cultured in IMDM medium supplemented 10% FBS, HL-60 and KG-1 cell lines were cultured in IMDM medium supplemented with 20% FBS, while AML-193 cell lines was cultured in IMDM medium supplemented with 5% FBS, 0.005 mg/ml insulin, 0.005 mg/ml transferrin and 5 ng/ml GM-CSF. All the cell lines were maintained in a

37°C humidified incubator with 5% CO_2. The cells were passaged every 2 to 3 days in order to maintain them in logarithmic growth phase. Cytarabine was purchased from Sigma Aldrich (St. Louise, MO) and drug dilutions were prepared with sterile water.

4.7.2. Drug Treatment: The AML cell lines, HL-60, MV-4-11, Kasumi-1, THP-1, AML-193, KG-1, MOLM-16 and ME-1 were grown till they reached 80% confluency. On the day of the drug treatment the cells were transferred in fresh medium and were plated in a 6-well plate at seeding density of 1.0×10^6 cells/ml. Cytarabine was added to the cells at a concentration of 0μM (baseline), 1μM or 10μM. The cells were then incubated at 37°C for 24h. The drug concentrations were chosen based on the steady state concentrations of the low dose and high dose cytarabine. After 24h, the cell pellets collected, washed with PBS and stored in -80°C till further use.

4.7.3. RNA Isolation: Total RNA was isolated from the AML cell pellets (baseline and cytarabine treated samples) using RNeasy Plus Mini Kit (QIAGEN, USA) according to the manufacturer's protocol and stored in -80°C until further analysis. The RNA quality and concentration was measured using NanoDrop 2000 UV-Vis spectrophotometer (ThermoScientific, USA). The ratio of absorbance at 260 nm and 280 nm was used to assess RNA sample purity and A260/A280 ratio of 1.8-2.1 was considered to be indicative of highly purified RNA. RNA was normalized to 0.2μg/μl with nuclease-free water before being used for performing reverse transcription reactions, as recommended by the manufacturer. The total RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to manufacturer's protocol.

4.7.4. Gene Expression Analysis: The change in expression of nucleoside analog genes after cytarabine treatment was determined using the TaqMan® Low Density Array (TLDA) cards (Applied Biosystems, USA). Each TLDA card was custom designed with pre-loaded gene expression assays for measuring the mRNA expression of selected nucleoside analog metabolic pathway genes (n =14) - deoxycytidine kinase (DCK), cytidine deaminase (CDA), solute carrier family 29, member 1 (SLC29A1), solute carrier family 28, member 1 (SLC28A1), solute carrier family 28, member 3 (SLC28A3), deoxycytidylate deaminase (DCTD), 5'-nucleotidase, cytosolic II (NT5C2), 5'-nucleotidase cytosolic III (NT5C3), cytidine 5'-triphosphate synthase (CTPS), cytidine monophosphate kinase (CMPK), nucleoside diphosphate kinase 1 (NME1), ribonucleotide reductase M1 (RRM1), ribonucleotide reductase M2 (RRM2), ribonucleotide reductase M2B (RRM2B). Each TLDA card consists of eight separate loading ports that fill into 48 separate wells, for a total of 384 wells per card. Thus, each card could analyze the expression of 24 different genes for 8 different samples in duplicates. Each cDNA sample was added to equal volume of 2X TaqMan Universal PCR Master Mix and 100 µl of the sample-specific PCR mix was added to the fill reservoir on the TLDA card. The card was centrifuged twice for 1 minute at 1200 rpm to distribute the sample-specific PCR reaction mix to the reaction wells. The card was sealed using the TaqMan Array Micro Fluidic Card Sealer and placed on microfluidic card thermal cycling block of Applied Biosystems 7900HT Fast Real-time PCR System (Applied Biosystems). Thermal cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 94.5°C, 30 seconds at 97°C, 1 minute at 59.7°C for 40 cycles. The target

mRNA expression levels were normalized to GAPDH and the change in the expression values of nucleoside analogs pathway genes after cytarabine treatment (1μM and 10μM) compared to the baseline gene expression was calculated using comparative C_T method [30].

4.7.5. MicroRNA Expression Analysis: For determination of change in microRNA expression after cytarabine treatment, total RNA was isolated using mirVana™ miRNA Isolation kit (Life Technologies, USA) as per the manufacturer's protocol. The RNA quality and concentration was measured using NanoDrop 2000 UV-Vis spectrophotometer (ThermoScientific, USA). A total of 100 ng of purified total RNA was used for nCounter miRNA sample preparation reactions according to manufacturer's instructions and was assayed for determination of 800 human microRNA expression using the nCounter Human v2 miRNA Expression Assay kit (Nanostring Technologies, USA). Preparation of small RNA samples involved multiplexed ligation of specific tags (miRtags) to the target microRNAs that provide unique identification for each microRNA species. After ligation, the detection was done by hybridization to microRNA: tag specific nCounter capture and barcoded reporter probes. Data collection was carried out using the nCounter Digital Analyzer (Nanostring Technologies, USA) at The University of Minnesota Genomics Center, following manufacturer's instructions to count individual fluorescent barcodes and quantify the target microRNA molecules present in each sample. MicroRNA expression data normalization was performed using the nSolver™ Analysis Software (Nanostring Technologies) according to the manufacturer's instructions. In particular, initially the data was normalization using the expression of the

top 100 codesets was used. Further, to account for the background correction, mean of negative controls plus two standard deviation (SD) method was used. In order to avoid using the microRNAs with a very low expression, we further filtered out the microRNAs with expression counts <30 (2 times the mean ± 2 SD of negative control value), in order to account for the background noise. Total 504 microRNAs with expression counts >30 at, either baseline, after 1μM or 10μM were included for further analysis.

4.7.6. Statistical Analysis: All values are expressed as ± standard deviation (SD). Effect of cytarabine treatment was compared by unpaired Student's t-test. Statistical analysis was performed using the GraphPad Prims software version 6.0 (GraphPad Software Inc., San Diego, CA). A difference was considered statistically significant when the probability was less than 0.05 ($p < 0.05$).

4.8. Results

4.8.1. Effect of cytarabine treatment on microRNA expression in AML cell lines:

Treatment of AML cell lines with cytarabine, at concentrations of either 1 μM or 10 μM, resulted in significant changes in expression of nearly 30 microRNAs. The observed changes in microRNA expression were different in sensitive cell lines (HL-60, MV-4-11, ME-1 and KG-1) and resistant cell lines (Kasumi-1, THP-1, AML-193 and MOLM-16) and were also concentration dependent.

In sensitive cell lines, expression of 22 microRNAs was significantly decreased post 1 μM cytarabine treatment as compared to the baseline microRNA expression (Figures 4B.1A and 4B.1B), while treatment at 10 μM of cytarabine significantly decreased the

expression of hsa-miR-4435, and increased the expression of hsa-miR-410 and hsa-miR-4508 in sensitive AML cell lines (Figure 4B.2). For hsa-miR-4508, there was ~ 40 % decrease in expression following 1 μM treatment while, the expression following treatment at 10 μM was ~ 2 fold higher. Expression of hsa-miR-4435 showed 2-fold decrease in expression after both 1 μM and 10 μM cytarabine treatments, indicating no concentration dependent effect (Figure 4B.1A and 4B.2).

In resistant cell lines the expression of 6 microRNAs was significantly decreased post 1 μM cytarabine (Figure 4B.3), while following treatment with 10 μM cytarabine only 3 microRNAs were significantly altered (Figure 4B.4). Interestingly, there was a concentration dependent decrease in expression of hsa-miR-605 with ~ 30 % decrease in expression following 1 μM treatment and ~ 40 % decrease following 10 μM treatment. These results indicate that the expression of microRNAs could be both induced or repressed post cytarabine treatment and this change in expression is dependent upon the concentration of cytarabine.

4.8.2. Effect of cytarabine treatment on mRNA expression of cytarabine pathway genes in AML cell lines: The effect of cytarabine treatment on the expression of cytarabine pathway genes was studied after incubation of AML cell lines with 1 μM and 10 μM cytarabine (Table 4B.1 and 4B.2, Figure 4B.5). Treatment with 1 μM cytarabine increased the expression of DCK, CDA and RRM2 mRNA greater than 2-fold in resistant AML cell lines (Kasumi-1, AML-193, THP-1 and MOLM-16) as compared to their respective mRNA expressions at baseline, while the expression of RRM1 and RRM2B was increased greater than 2-fold in sensitive AML cell lines (HL-60, MV-4-11, KG-1

and ME-1) as compared to their mRNA expressions at the baseline (Table 4B.1 and 4B.2, Figure 4B.5). Interestingly treatment with 10 μM cytarabine resulted in greater 2-fold increase in expression of DCK, CDA and RRM2 in both sensitive and resistant cell lines (Figure 4B.5). Expression of CDA, which is an inactivating gene in the cytarabine metabolic pathway, demonstrated a concentration dependent increase after 10 μM cytarabine treatment in resistant AML cell lines. Expression of RRM1, RRM2B and CTPS1 increased only in cytarabine-sensitive cell lines after 10 μM cytarabine treatment (Table 4B.1).

4.9. Discussion:

There is increasing evidence that chemotherapy treatment affects the expression of various genes [24, 25, 31-34]. Recently, many studies have also identified changes in the expression patterns of various microRNAs post treatment with various anticancer agents [27-29, 35]. However, not many studies have studied the effect of cytarabine treatment on the expression changes of various microRNAs as well as the expression changes of the metabolic pathway genes of cytarabine. Since microRNAs are known to modulate the expression of various drug metabolizing enzymes and/or transporters, changes in microRNA expression post cytarabine treatment can potentially lead to alteration in the expression of the target genes of these microRNAs, thereby affecting their biological function. Such information could be important to further elucidate the mechanisms of acquired resistance to cytarabine treatment. In addition, understanding the effect of cytarabine treatment on the expression of its metabolic pathway genes could additionally

provide an increased understanding of the effect of cytarabine treatment on its own activation/inactivation pathway.

In this study we determined the effect of cytarabine treatment expression of microRNAs (n=800) and PK/PD pathway genes (n=14) of cytarabine in AML cell lines. We evaluated the effect of two concentrations, 1 μM and 10 μM. We selected to test these concentrations of cytarabine based on the steady state concentrations (Cp, ss) achieved with standard dose cytarabine (100-200 mg/m^2) (Cp, ss = 0.5 -1 μM) [36, 37] and high dose cytarabine (2-3 gm/m^2) (Cp, ss ~ 10 to 50 μM) [38, 39]. We identified 22 microRNAs that were significantly downregulated in AML cell lines (HL-60, MV-4-11, KG-1 and ME-1) sensitive to 1 μM cytarabine treatment, while treatment with 10 μM cytarabine increased the expression of hsa-miR-410 and hsa-miR-4508 and decreased the expression of hsa-miR-4435 in sensitive AML cell lines. In resistant AML cell lines (Kasumi-1, THP-1, AML-193 and MOLM-16) treatment with 1 μM cytarabine resulted in decreased expression of 6 microRNAs, while 10 μM cytarabine treatment decreased the expression of hsa-miR-769-3p and hsa-miR-605, while expression of hsa-miR-643 was increased. Our data reveals that cytarabine treatment can result in differential effects on microRNA expression in AML cell lines. Out of the microRNAs affected by cytarabine treatment, a few demonstrated concentration dependent effect. In resistant cell lines, microRNA hsa-miR-605 resulted in ~ 30% decrease in expression following treatment with 1 μM cytarabine and ~ 40% decrease in expression following 10 μM cytarabine treatment. MicroRNA hsa-miR-605 expression has been implicated in inducing apoptosis by activation of p53 expression by directly repressing Mdm2

oncoprotein, an E3 ubiquitin ligase, which negatively regulates p53 by blocking p53 mediated transactivation (p53: Mdm2 negative feedback loop) [40, 41]. Thus, cytarabine-induced downregulation in the expression of hsa-miR-605 could potentially affect the tumor suppressive activity of p53. Expression of hsa-miR-4435 in sensitive cell lines was consistently decreased after cytarabine treatment with 1 μM and 10 μM, without demonstrating any concentration dependent effect. These results indicate that the effect of cytarabine treatment could lead to differential effects on the expression of different microRNAs in AML cell lines. Interestingly, in sensitive cell lines, the expression of hsa-miR-4508 was decreased ~ 2.5-fold after 1 μM cytarabine treatment; however there was ~ 2-fold increase in expression of this microRNA after 10 μM cytarabine treatment. Though the biological relevance of hsa-miR-4508 has not been identified, this novel regulation of hsa-miR-4508 by cytarabine warrants further investigation. Thus, our data reveals that cytarabine treatment leads to significant change in microRNA expression; however, whether this alteration in microRNA expression would lead to significant alteration of microRNA targets and their biological functions requires additional investigation.

In addition, we also identified the effect of cytarabine treatment on the expression of PK/PD pathway genes of cytarabine in AML cell lines. Post-cytarabine treatment, DCK expression was increased 2-fold in resistant cell lines following 1 μM treatment and 2.5-fold and 2-fold in both sensitive and resistant cell lines, respectively following 10 μM treatment. Though the exact mechanism of this alteration in the activating enzyme in the cytarabine pathway awaits further investigations, one of the mechanisms of this

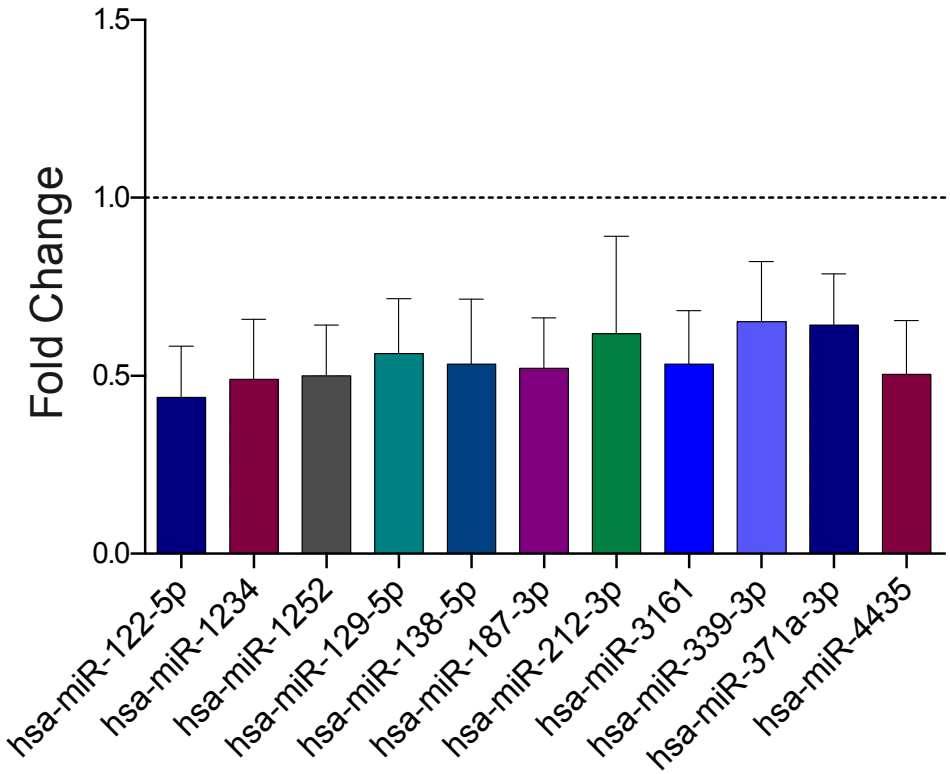
regulation could be by microRNAs. Interestingly, in our previous study (Chapter III), we identified that the expression of DCK (the rate-limiting enzyme in the nucleoside analog pathway) was negatively correlated with the expression of hsa-miR-34a-5p (spearman r = -0.881; p-value < 0.01). Cytarabine treatment also affected the expression of hsa-miR-34a-5p, with 1 μM cytarabine significantly decreasing the expression of hsa-miR-34a-5p in resistant cell lines. Even though there was a decrease in hsa-miR-34a-5p expression in sensitive cell lines post 1 μM and 10 μM and in resistant cell lines post 10 μM cytarabine treatment, the decrease in expression was not found to be statistically significant. Hence the repression of hsa-miR-34a-5p expression post cytarabine treatment could partly explain the increase in DCK expression. Cytarabine treatment was also found to significantly alter the expression of the primary deactivating enzyme, CDA and ribonucleotide reductases, indicating the importance of understanding the effect of cytarabine treatment on the activation/inactivation pathway of cytarabine.

In conclusion, we provided preliminary data to demonstrate the effect of cytarabine treatment on the expression of microRNAs and cytarabine PK/PD pathway genes, demonstrating that the expression of microRNAs and mRNAs could be affected by chemotherapy. Our results would require further verification and validation, in both *in vitro* as well as *in vivo* settings to elucidate the mechanism effect of cytarabine. Also, since the primary aim of the current study was to understand the effect of cytarabine treatment on the expression of microRNAs (and cytarabine pathway genes), we did not identify the target mRNAs for the differentially expressed microRNAs affected by cytarabine treatment. Future studies would be focused on identifying the specific targets

of the microRNAs altered by cytarabine treatment and investigating the important biological pathways affected by deregulated microRNAs by cytarabine treatment.

The current study in AML cell lines also helped us establish an *in vitro* system to validate the important findings from the *in vivo* study conducted to understand effect of cytarabine treatment in AML patient samples (Chapter IV-A). Thus, we demonstrated that cytarabine treatment in AML cell lines leads to changes in microRNA and PK/PD pathway gene expression after both 1 μM and 10 μM treatments. Identification of the underlying mechanism of modulation of microRNA and mRNA expression would ultimately provide increased understanding of the effect of cytarabine treatment and development of acquired resistance, thereby helping to improve the treatment outcomes.

A



Figures 4B.1: (A) Change in microRNA expression in sensitive AML cell lines after 1 μM Cytarabine treatment

B

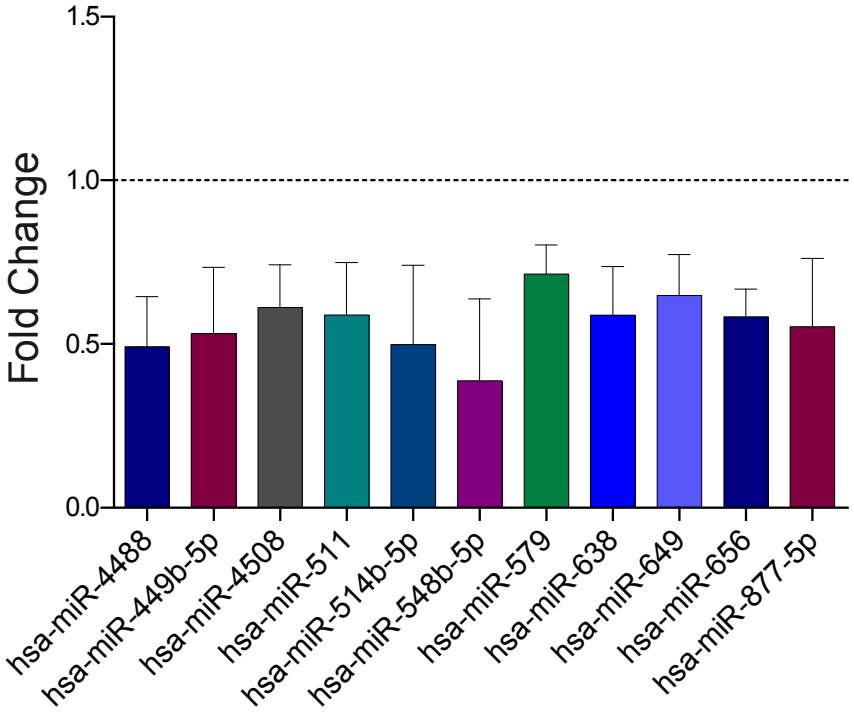


Figure 4B.1: (B) Change in microRNA expression in sensitive AML cell lines after 1 μM Cytarabine treatment

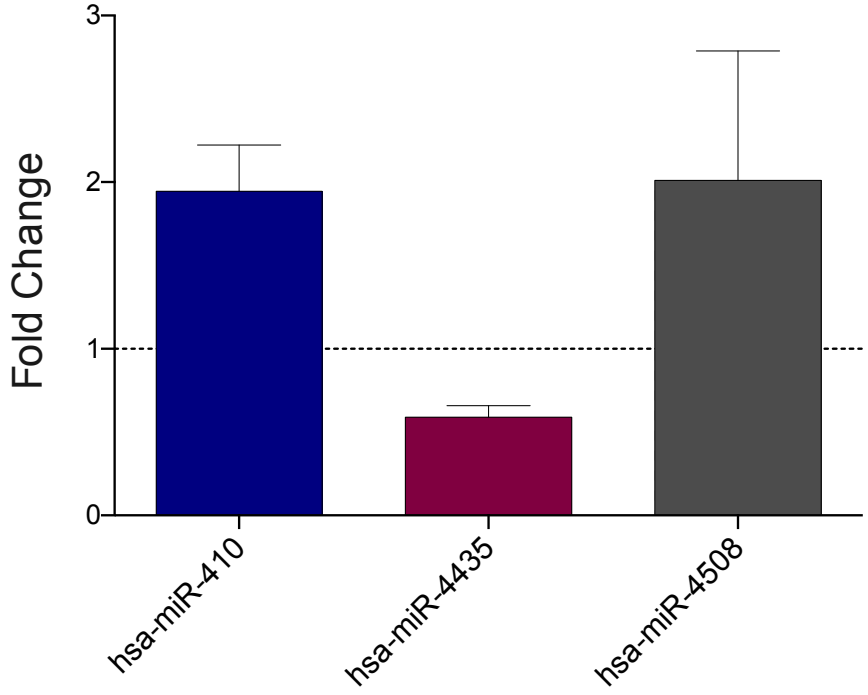


Figure 4B.2: Change in microRNA expression in sensitive AML cell lines after 10 μM Cytarabine treatment

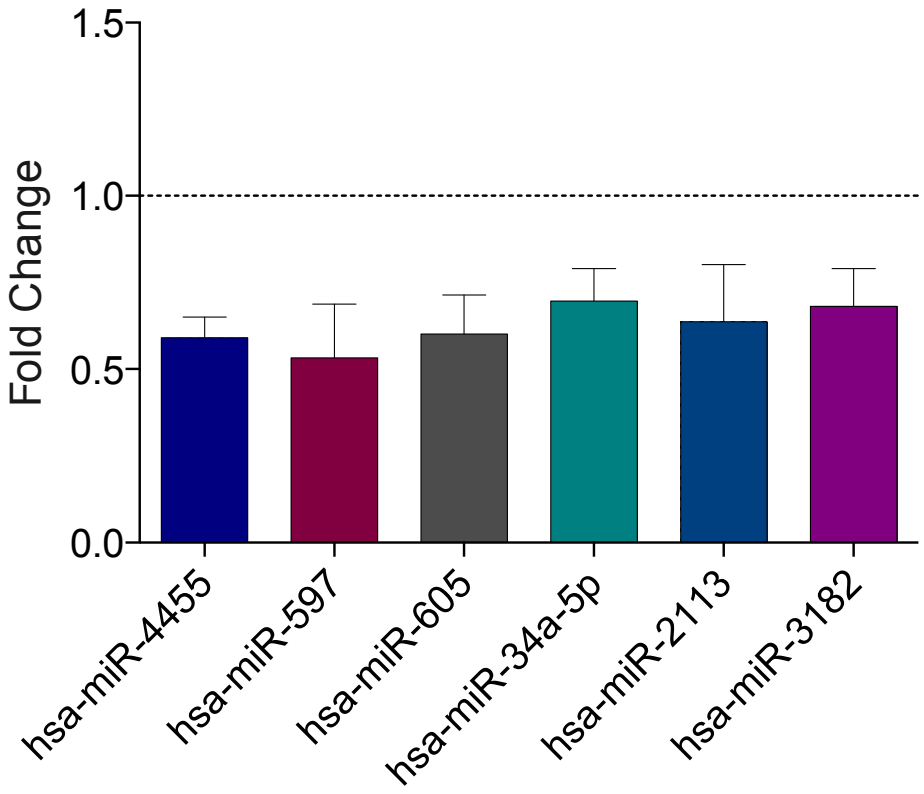


Figure 4B.3: Change in microRNA expression in resistant AML cell lines after 1 μM Cytarabine treatment

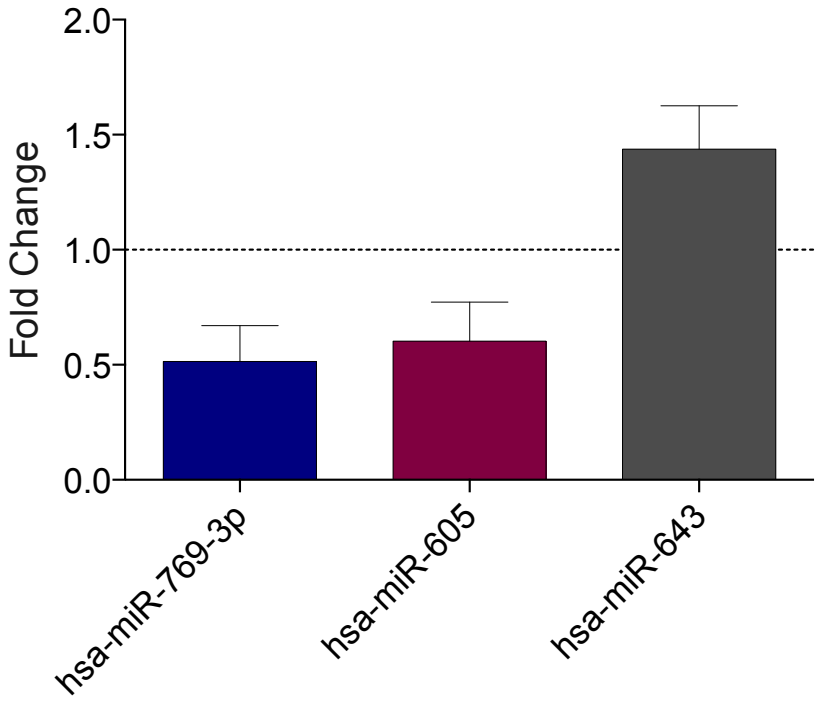


Figure 4B.4: Change in microRNA expression in resistant AML cell lines after 10 μM Cytarabine treatment

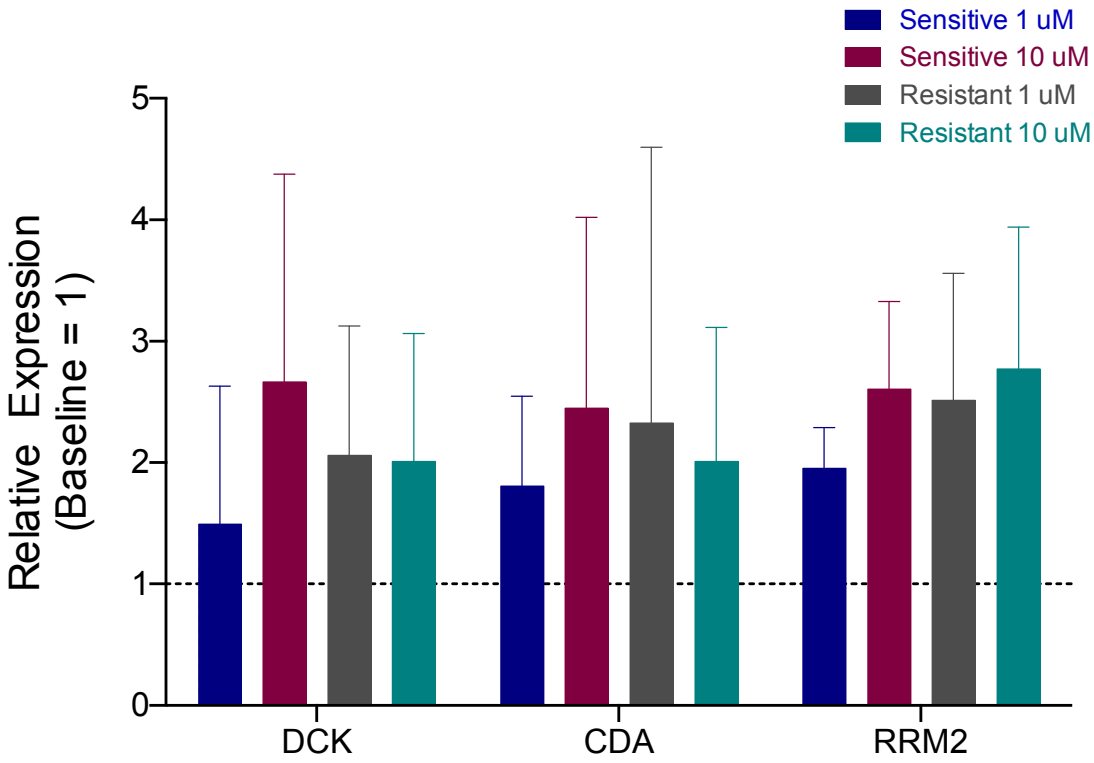


Figure 4B.5: Change in mRNA expression in AML cell lines after 1 μM and 10 μM Cytarabine treatment

Table 4B.1: Change in mRNA Expression of pathway genes in cytarabine sensitive AML cell lines post cytarabine treatment (1 μM and 10 μM)

Genes	Sensitive (mRNA Expression)			
	1 μM Treatment		10 μM Treatment	
	Mean	SD	Mean	SD
DCK	1.49	1.13	2.66	1.71
SLC29A1	1.44	0.32	1.84	0.70
CDA	1.81	0.74	2.45	1.57
NT5C2	1.48	0.21	1.79	0.44
NT5C3	0.97	0.36	1.26	0.39
RRM1	2.05	0.67	2.18	0.85
RRM2	1.95	0.34	2.60	0.72
RRM2B	6.37	8.67	3.11	3.33
CTPS1	1.29	0.39	2.39	2.04
CMPK1	1.15	0.59	1.41	0.82
DCTD	1.25	0.59	1.24	0.47
NME1	1.08	0.43	2.68	2.06

Table 4B.2: Change in mRNA Expression of pathway genes in cytarabine resistant AML cell lines post cytarabine treatment (1 μM and 10 μM)

Genes	Resistant (mRNA Expression)			
	1 μM Treatment		10 μM Treatment	
	Mean	SD	Mean	SD
DCK	2.06	1.07	2.01	1.05
SLC29A1	1.54	0.57	1.48	0.53
CDA	2.33	2.27	5.09	5.39
NT5C2	1.39	0.32	1.52	0.14
NT5C3	1.19	0.51	1.49	0.47
RRM1	1.19	0.48	1.57	0.58
RRM2	2.52	1.04	2.77	1.16
RRM2B	1.65	0.94	1.41	0.45
CTPS1	1.37	0.37	1.32	0.36
CMPK1	1.03	0.30	1.60	0.79
DCTD	1.01	0.42	0.95	0.26
NME1	1.13	0.53	0.98	0.35

CHAPTER V

MicroRNAs associated with Clofarabine Cytotoxicity in Acute Myeloid Leukemia

5.1. Introduction

Acute myeloid leukemia (AML) is a hematological malignancy affecting the myeloid progenitor cells in the bone marrow. AML remains the most common form of leukemia in adults and the second most common form of leukemia in children. However, despite the recent advances in research and treatment strategies, AML continues to have poor survival rate [1]. Cytarabine, which was first approved by FDA in the late 1960s, in combination with anthracycline remains the cornerstone of treatment for AML. Cytarabine-based induction regimens are known to induce complete remission (CR) in about 60% to 80% of younger AML patients (≤ 55-60 years) and long-term disease free survival is around 30% [2, 3], while in older AML patients (≥ 55-60 years) the complete remission rate is 40% to 55%, with a poor long-term survival rate (10% to 15%) [4]. One of the strategies to improve the outcome involves addition of other nucleoside analogs, specifically purine nucleoside analogs, to the cytarabine-based induction regimen [5-7]. In particular, combination of fludarabine with cytarabine and idarubicin has been shown to result in significantly better complete remission rate (74%) and less hematologic and non-hematologic toxicity; as compared to cytarabine, idarubicin and etoposide treatment arm (CR of 51%) [5]. Similarly, inclusion of cladribine to the 3+7 regimen of cytarabine and daunorubicin was found to result in significantly higher CR rates (64%) compared the standard treatment arm comprising of cytarabine and daunorubicin (47%) [6]. Treatment of relapsed and refractory AML presents another challenge, since about 20% to 40% of AML patients fail to achieve complete remission following standard induction regimen, and 50% to 70% of who achieve CR are expected to relapse within 3 years [8,

9]. In addition, once the patient has had a relapse with the current therapies their chances of long-term disease free survival is less than 10% [10]. Since there is no single standard treatment regimen, it is essential to identify new combinations of standard chemotherapy regimens or novel agents for the treatment of relapsed or refractory disease. Some prospective clinical trials have evaluated the significance of purine nucleoside analogs, specifically fludarabine and cladribine in combination with cytarabine for the treatment of relapsed or refractory disease [11-14].

Fludarabine and cladribine when used in combination with cytarabine are known to potentiate the cytotoxicity of cytarabine by increasing the accumulation of its triphosphate metabolite [11, 15, 16]. The main drawback of cladribine and fludarabine is their low oral bioavailability due to their instability in gastric pH and susceptibility to bacterial cleavage [17]. In addition, both these purine analogs have been associated with moderate myelosuppression and profound and prolong immunosuppression, with high doses leading to neurological toxicities [18]. However, the promising results observed in clinical studies with combination of fludarabine or cladribine with cytarabine, provided rationale for investigation of other purine nucleoside analogs.

Clofarabine is a second-generation purine nucleoside analog, designed specifically to incorporate the favorable characteristics of fludarabine and cladribine and to overcome the dose-limiting adverse effects associated with these drugs. Structural modifications in clofarabine result in resistance to deamination by adenosine deaminase and decrease its susceptibility to phosphorolytic cleavage, leading to enhanced oral bioavailability of clofarabine [19, 20]. Structural activity relationships demonstrate that clofarabine

incorporates mechanistically favorable properties of both fludarabine and cladribine, such as inhibition of DNA polymerases (like fludarabine) and inhibition of ribonucleotide reductases (like cladribine) [21-25]. In fact, clofarabine appears to have greater affinity for dCK, exceeding fludarabine and cladribine [19, 26]. Clofarabine and cladribine are superior to fludarabine in inhibiting ribonucleotide reductase while clofarabine and fludarabine are more efficient than cladribine in inhibition of DNA polymerase [27]. In addition, clofarabine is known to induce apoptosis by releasing pro-apoptotic mitochondrial factor, which was absent in fludarabine. Interestingly, the rate-limiting step in activation of clofarabine triphosphate is the conversion of clofarabine monophosphate to diphosphate by purine monophosphate kinase, in contrast to other nucleoside analogs, where formation of monophosphate form by DCK is the rate-limiting step [24]. Clofarabine, due to its favorable PK/PD is preferred over the currently used fludarabine and cladribine and has recently been investigated in multiple Phase I and Phase II clinical trials as a chemotherapeutic agent for hematological malignancies (clinical trials.gov study identifiers: NCT00067028, NCT00334074, NCT01025154 etc.). Use of clofarabine as a single agent established the significance of this second-generation purine analog for treatment of AML [28, 29]. Initial studies combining fludarabine or cladribine with cytarabine had shown promising results, which prompted the efforts to study the combination regimens of cytarabine and clofarabine. In addition, *in vitro* studies in leukemic cell lines, had demonstrated the synergy between cytarabine and clofarabine, with pre-incubation with clofarabine increasing the cytarabine triphosphate levels by two-fold [25, 30]. The promising results from the preclinical studies as well as from the initial

trials using clofarabine as a single agent, led to investigation of clofarabine in combination with cytarabine [31-33]. Phase I-II trial in relapsed and refractory AML patients investigated combination of clofarabine with cytarabine in an effort to modulate cytarabine triphosphate accumulation [31]. This study reported complete response (CR) in 22% patients with an overall response (OR) rate of 38%. Phase II study in previously untreated adult AML patients conducted to assess the efficacy of clofarabine and cytarabine combination demonstrated an OR rate of 60%, with 52% CR in older AML patients [34]. A randomized study comparing clofarabine plus cytarabine versus clofarabine alone also showed promising results [33]. The encouraging results from these trials resulted in additional combination of clofarabine and cytarabine with anthracyclines. Phase I study with primary refractory or first-relapse AML, combination of clofarabine with cytarabine and idarubicin showed a high response rate compared to the cytarabine plus idarubicin [35]. Although clofarabine has been currently shown to be a promising agent for the treatment of AML, there are no studies evaluating the significance of microRNA expression in clofarabine cytotoxicity. Our previous studies with cytarabine, which shares the PK/PD pathway with clofarabine, have shown that microRNAs regulate the expression of genes in PK/PD pathway of clofarabine. Also, microRNA deregulation has been implicated in AML pathogenesis and several studies have demonstrated the significance of microRNAs in predicting outcome in AML patients [36-40]. Since minor structural differences in clofarabine result in significant differences in the activity as well as the metabolic and pharmacokinetic profile as compared to cytarabine, an independent analysis to study the effect of microRNAs on

clofarabine response seems like a reasonable approach. In this study, we report the results from genome-wide microRNA expression analysis to identify the microRNAs associated with clofarabine cytotoxicity in eight AML cell lines.

5.2. Materials and Methods

5.2.1. Cell Culture and Reagents: The AML cell lines HL-60, MV-4-11, Kasumi-1, THP-1, AML-193 and KG-1 cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA), while MOLM-16 and ME-1 cell lines were obtained from DSMZ (Braunschweig, Germany). Kasumi-1, ME-1 and MOLM-16 cell lines were cultured in RMPI-1640 medium supplemented with 20% fetal bovine serum (FBS), THP-1 cell line was cultured in RPMI-1640 medium supplemented with 10% FBS, MV-4-11 cell line was cultured in IMDM medium supplemented 10% FBS, HL-60 and KG-1 cell lines were cultured in IMDM medium supplemented with 20% FBS, while AML-193 cell lines was cultured in IMDM medium supplemented with 5% FBS, 0.005 mg/ml insulin, 0.005 mg/ml transferrin and 5 ng/ml GM-CSF. All the cell lines were maintained in a 37°C humidified incubator with 5% CO_2. The cells were passaged every 2 to 3 days in order to maintain them in logarithmic growth phase. Clofarabine was purchased from Sigma Aldrich (St. Louis, MO).

5.2.2. Cytotoxicity Assay: Clofarabine cytotoxicity was determined using the MTT assay. Clofarabine (Sigma Aldrich) solution was prepared in dark by dissolving clofarabine powder in DMSO to obtain a stock concentration of 10 mg/ml. AML cell lines were plated in 96-well plate at seeding density of 2.5×10^5 cells/ml and incubated at

37°C overnight. After 24h of recovery time, the cells were exposed to varying concentrations (5, 2.5, 1, 0.5, 0.05, 0.025 and 0 μM) of clofarabine. Cell viability was determined 48h post clofarabine treatment by MTT assay by measuring the absorbance at 570nm using Synergy plate reader (BioTek, USA). The percent cell survival at each concentration was calculated using the Gen5™ Software version 1.11 (Winooski, VT). The area under the survival curve (AUC) was calculated by the trapezoidal method using the GraphPad Prism software version 6 (La Jolla, California). Each cytotoxicity experiment was repeated at least 3 times.

5.2.3. Apoptosis Assay: The apoptotic activity of AML cell lines following treatment with varying concentration of clofarabine (as indicated above) was determined using the Caspase-Glo® 3/7 assay as per manufacturer's instructions (Promega, USA). 48h post clofarabine treatment, luminescence was read using Synergy plate reader (BioTek, USA). The luminescence produced is directly proportional to the caspase activity. The caspase activity at each concentration was normalized to the control and the area under the relative caspase activity curve (AUC) was calculated by the trapezoidal method using the GraphPad Prism software version 6 (La Jolla, California). Each caspase experiment was repeated 3 times.

5.2.4. MicroRNA Expression Analysis: For determination of microRNA expression, total RNA was isolated using mirVana™ miRNA Isolation kit (Life Technologies, USA) as per the manufacturer's protocol. The RNA quality and concentration was measured using NanoDrop 2000 UV-Vis spectrophotometer (ThermoScientific, USA). A total of 100 ng of purified total RNA was used for nCounter miRNA sample preparation

reactions according to manufacturer's instructions and the expression of 800 human microRNAs was quantified using the nCounter Human v2 miRNA Expression Assay kit (Nanostring Technologies, USA). Preparation of small RNA samples involved multiplexed ligation of specific tags (miRtags) to the target microRNAs that provide unique identification for each microRNA species. After ligation, the detection was done by hybridization to microRNA: tag specific nCounter capture and barcoded reporter probes. Data collection was carried out using the nCounter Digital Analyzer (Nanostring Technologies, USA) at The University of Minnesota Genomics Center, following manufacturer's instructions to count individual fluorescent barcodes and quantify the target microRNA molecules present in each sample. MicroRNA expression data normalization was performed using the nSolver™ Analysis Software (Nanostring Technologies) according to the manufacturer's instructions. In particular, initially the data was normalization using the expression of the top 100 codesets was used. Further, to account for the background correction, mean of negative controls plus two standard deviation (SD) method was used. In order to avoid using the microRNAs with a very low expression, we further filtered out the microRNAs with expression counts <30 (2 times the mean ± 2 SD of negative control value), in order to account for the background noise. Total 412 microRNAs with expression counts >30 were evaluated for differential expression between sensitive and resistant cell lines and for their correlation with cytarabine chemosensitivity.

5.2.5. Statistical Analysis: The differential expression of microRNAs between the sensitive and resistant AML cell lines to clofarabine was determined by using Student's t-

test of the log-transformed data. Nonparametric Spearman correlation was used to measure the correlation of AUCs with microRNA expression. All statistical analysis was performed using Graph Pad Prism software. Heat map was generated using the NSolver software.

5.3. Results

5.3.1. Clofarabine chemosensitivity in AML cell lines: The overall study design is shown in Figure 5.1. We estimated *in vitro* clofarabine-induced cytotoxicity and clofarabine-induced apoptosis in 8 cytogenetically different AML cell lines in order to characterize clofarabine response in different AML cell lines. As, expected we observed variation in AML clofarabine cell cytotoxicity AUC, with mean AUC value of 151.96 (±96.56) (Figure 5.2A). We observed that MV-4-11 cell line was the most sensitive to clofarabine and Kasumi-1 cell lines was the most resistant. Cell lines MV-4-11, HL-60, THP-1 and KG-1 were classified as sensitive cell lines (AUC <150), while MOLM-16, ME-1, AML-193 and Kasumi-1 were classified as resistant cell lines (AUC > 150) (Table 5.1). Induction of apoptosis as measured by caspase 3/7 activity with increasing concentration of clofarabine was complimentary to the observed cell viability; percent cell survival after each increasing concentration of clofarabine decreased as the apoptotic activity for the respective concentration increased (Figure 5.3A to 5.3H).

5.3.2. Association of microRNAs with clofarabine chemosensitivity in AML cell lines: In order to understand the effect of microRNAs in the chemosensitivity of AML cell lines to clofarabine, we performed global microRNA expression profiling of the

AML cell lines. We examined the expression of 800 human microRNAs in these cell lines using nCounter Nanostring platform. After normalization and background correction we filtered out microRNAs with expression counts <30 (2 times the mean ± 2 SD of negative control value), in order to account for the background noise. Total 412 microRNAs with expression counts >30 were evaluated for differential expression between sensitive and resistant cell lines and for their correlation with clofarabine chemosensitivity. The heat map shows the top 25 differentially expressed microRNAs between the sensitive and resistant cell lines (Figure 5.4B) based on cell viability assays. Higher levels of hsa-miR-335-5p, hsa-miR-1290, hsa-miR-3690, hsa-miR-486-3p, hsa-miR-525-5p, hsa-miR-148b-3p, hsa-miR-194-5p, hsa-miR-331-3p and lower levels of hsa-miR-16-5p, hsa-miR-423-3p, hsa-miR-30b-5p and hsa-miR-548aa were associated with clofarabine resistance, p-value < 0.05 as depicted in the volcano plot (Figure 5.5). Using cell sensitivity phenotype as a continuous variable, we identified 25 unique microRNAs significantly associated with clofarabine-induced cytotoxicity and apoptosis AUC (Table 5.2). We identified that cluster of miR-515 family members were positively associated with clofarabine-induced cytotoxicity. This microRNA miR-515 cluster along with some other microRNAs was also found to be upregulated in cisplatin resistant germ cell tumor cell lines compared to the parenteral cell lines [41]. Of the differentially expressed microRNAs, hsa-miR-16-5p was found to be significantly overexpressed in clofarabine-sensitive cell lines as compared to the resistant cell lines. Interestingly, the microRNA hsa-miR-16-5p was also found to be negatively associated with clofarabine-induced cytotoxicity AUC, indicating increased sensitivity towards clofarabine in cells

expressing low levels of hsa-miR-16-5p. On the other hand, higher levels of microRNAs hsa-miR-194-5p, hsa-miR-148b-3p, hsa-miR-486-3p, hsa-miR-525-5p, hsa-miR-331-3p and hsa-miR-1290 were positively associated with clofarabine resistance.

5.4. Discussion:

In this study, we identified the microRNAs that were associated with the clofarabine chemosensitivity in AML cell lines. We screened for global microRNA expression profiles in AML cell lines and identified 30 unique microRNAs associated with clofarabine chemosensitivity, among which miR-515 cluster was found to be significantly associated with clofarabine cytotoxicity. We also identified 8 microRNAs (hsa-miR-335-5p, hsa-miR-1290, hsa-miR-3690, hsa-miR-486-3p, hsa-miR-525-5p, hsa-miR-148b-3p, hsa-miR-194-5p and hsa-miR-331-3p), which showed increased expression in clofarabine sensitive AML cell lines and 4 microRNAs (hsa-miR-16-5p, hsa-miR-423-3p, hsa-miR-30b-5p and hsa-miR-548aa), which showed increased expression in clofarabine resistant AML cell lines.

With the increasing interest in clofarabine's therapeutic utility as demonstrated in the ongoing clinical trials, it is important to identify factors that might affect clofarabine's efficacy as a chemotherapeutic agent for the treatment of AML and other hematological malignancies. To our knowledge, there has been only one study demonstrating the potential impact of genetic polymorphisms (SNPs) and epigenetic modifications on clofarabine cytotoxicity in lymphoblastoid cell lines [42]. This study demonstrated the impact of integrating the epigenetic information, like DNA methylation with genotype

and gene expression information, in order to identify the genes relevant to clofarabine cytotoxicity. Using a comprehensive association analysis, this study identified six genes associated with clofarabine-mediated cytotoxicity. However the influence of microRNAs on clofarabine cytotoxicity was not investigated. Recent evidence indicates the importance of microRNAs in regulating the expression of pharmacologically relevant genes affecting the drug responses. Kovalchuk and colleagues identified that microRNA-451 regulates the expression of multidrug resistance 1 (MDR1) gene, in turn regulating the cellular resistance to doxorubicin in breast cancer cell lines [43]. MicroRNAs have also been demonstrated to regulate the expression of important drug metabolizing enzymes like cytochrome P450s [44, 45]. In addition to regulation of gene and/or protein expression, microRNAs have also shown to play an important role various malignancies, including AML. In our previous study (Chapter II), we identified microRNAs associated with cytarabine chemosensitivity and overall survival in AML patients. Hence, it is essential to understand the role of these significant class of regulators in contributing to the chemotherapeutic response to clofarabine. Even though we have shown the effect of microRNAs on cytarabine cytotoxicity (Chapter II), there is evidence, which shows that despite the structural similarities between clofarabine and cytarabine, there are differences in chemosensitivity to these nucleoside analogs in patients as well as in cell lines. For instance, sensitivity of acute lymphoblastic leukemia cell lines of B-lineage, were found to be more than 7-fold sensitive to clofarabine compared to cytarabine, while the T-lineage cells were not differentially affected [46]. Also, cytarabine-resistant leukemia cells were found to be moderately sensitive to clofarabine, and these differences

were attributed to the human concentrative nucleoside transporter 3 (hCNT3) which is involved in the influx of clofarabine in addition to human equilibrative transporters (hENT) 1 and 2, while cytarabine transport is mainly by hENT1 [47]. In addition, studies on nucleoside analog transport, revealed differences in hENT1 transport efficiency towards clofarabine, cytarabine and other nucleoside analogs [48]. This necessitates the importance to independently identify the role of microRNAs in predicting response to clofarabine.

In an effort to better understand the contribution of microRNAs in clofarabine-mediated chemosensitivity, screened for microRNA expression (n=800) in cytogenetically different AML cell lines and identified the microRNAs significantly associated with clofarabine-induced cytotoxicity and apoptosis in AML cell lines. We identified 25 microRNAs to be associated with clofarabine cell viability and apoptosis AUCs (p-value < 0.05), out of which 6 microRNAs were also differentially expressed between the AML cell lines sensitive and resistant to clofarabine. MicroRNA hsa-miR-16-5p, which was found have increased expression in clofarabine sensitive AML cell lines, was also found to be negatively associated with clofarabine-induced cytotoxicity AUC. In our previous study (Chapter II), we identified hsa-miR-16-5p was also predictive of cytarabine sensitivity in AML cell lines. Interestingly, miR-16 is located in the miR-15a/miR-16 cluster that is located at chromosome 13q14.3, a genomic region that is known to be frequently deleted in B cell chronic lymphocytic leukemia (B-CLL) [49]. Expression of miR-16 along with miR-15 inhibited leukemic cell proliferation and induced apoptosis by targeting multiple oncogenes such as Bcl-2, WNT3A, MCL1, and CND1, both *in vitro* and *in vivo* [50, 51].

Significance of miR-15/miR-16 cluster as tumor suppressors in leukemic cells could be due to their ability to down regulate of the expression of Wilms' tumor gene (WT1) oncogene [52]. These data show the significance of miR-16 in leukemia. Our study identified the significance of this important microRNA in clofarabine sensitivity. Additional studies to elucidate the exact mechanism of the role of miR-16 in nucleoside analog sensitivity are warranted.

We also identified microRNAs hsa-miR-194-5p, hsa-miR-148b-3p, hsa-miR-486-3p, hsa-miR-525-5p, hsa-miR-331-3p and hsa-miR-1290 to have increased expression in clofarabine resistant AML cell lines, in addition to being positively associated with clofarabine-induced cytotoxicity AUC. Thus, indicating that increased expression of these microRNAs could potentially contribute to increased clofarabine resistance. MicroRNA hsa-miR-486 has been reported earlier to be selectively expressed in Down's syndrome associated AML (DS-AML) compared to non-DS megakaryocytic leukemias (AMKLs), and expression of miR-486 is regulated by GATA1 binding [53]. It was also demonstrated that miR-486 expression contributes to the survival of CML progenitor cells after treatment with BCR-ABL tyrosine kinase inhibitor (TKI) imatinib mesylate, and inhibition of miR-486 significantly enhanced imatinib-mediated apoptosis [54]. Recent study demonstrated the role of increased expression of miR-331 in predicting worse response to therapy and decreased survival of AML patients [55] which is in accordance with our results which show increased expression of miR-331 associated increased resistance to clofarabine. Our study characterized the role of these and more important microRNAs in clofarabine response.

Interestingly, we also identified a cluster of miR-515 family members to be positively associated with clofarabine-induced cytotoxicity. MicroRNAs hsa-miR-519c and hsa-miR-520h, miR-515 family members, have been previously validated to be targeting and repressing the expression of ABCG2 efflux transporter [56]. This study demonstrated that ABCG2-overexpressing resistant subline S1M1 80 cell lines have shorter 3'UTR compared to the S1 parental cell line, thereby disrupting the hsa-miR-519c and hsa-miR-520h binding sites. Recent evidence indicates the role of ATP-binding cassette transporter, ABCG2 in playing a role in the efflux of purine nucleoside analogs, like clofarabine [57, 58]. In addition, expression of DCK and ABCG2 was shown to impact clofarabine metabolism and cytotoxicity [59]. Our results identified expression of miR-515 cluster predictive of clofarabine chemoresistance, which could partly be explained by the effect miR-515 family members on clofarabine metabolic pathway.

In conclusion, our study identified microRNAs relevant to clofarabine response in AML. To our knowledge, this is a first study characterizing significant microRNAs relevant to clofarabine cytotoxicity in AML. Additional functional validation and mechanistic studies are warranted to elucidate the role of these microRNAs in clofarabine response. Also validating these findings in AML patients would strengthen the significance of these microRNAs and help in better understanding the factors contributing to the response of these chemotherapeutic agents.

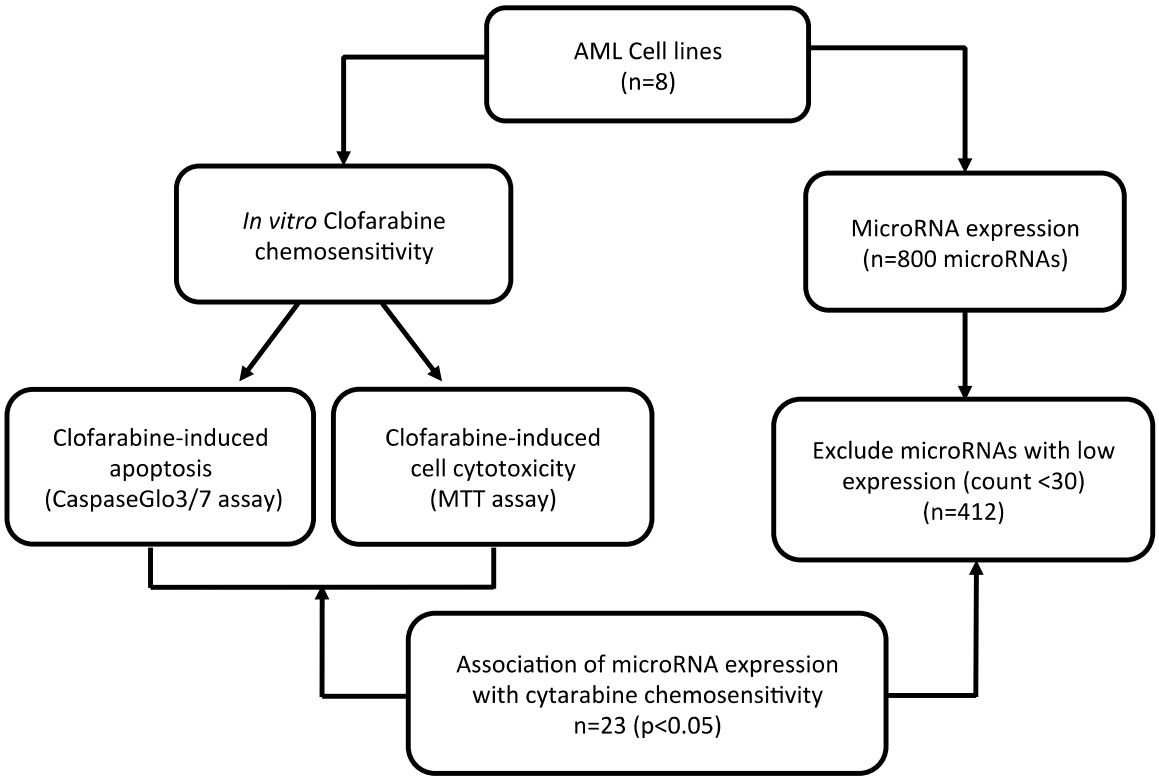


Figure 5.1: Overall schema of the proposed study

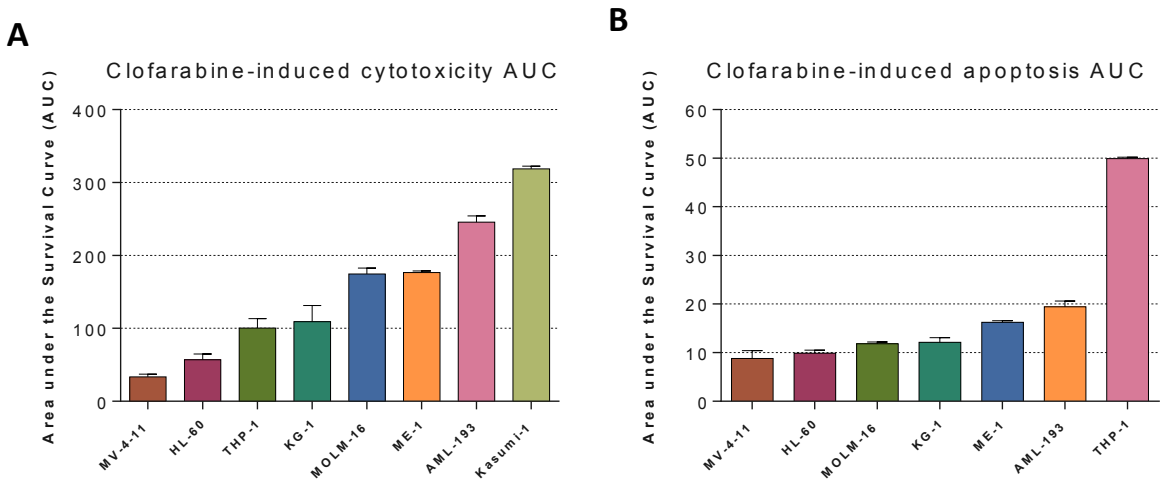


Figure 5.2: **Clofarabine-induced chemosensitivity AUC in AML cell lines** (A) Clofarabine-induced cytotoxicity area under the survival curve (AUC) of 8 AML cell lines after clofarabine treatment. (B) Clofarabine-induced apoptosis area under the survival curve (AUC) of 8 AML cell lines after clofarabine treatment.

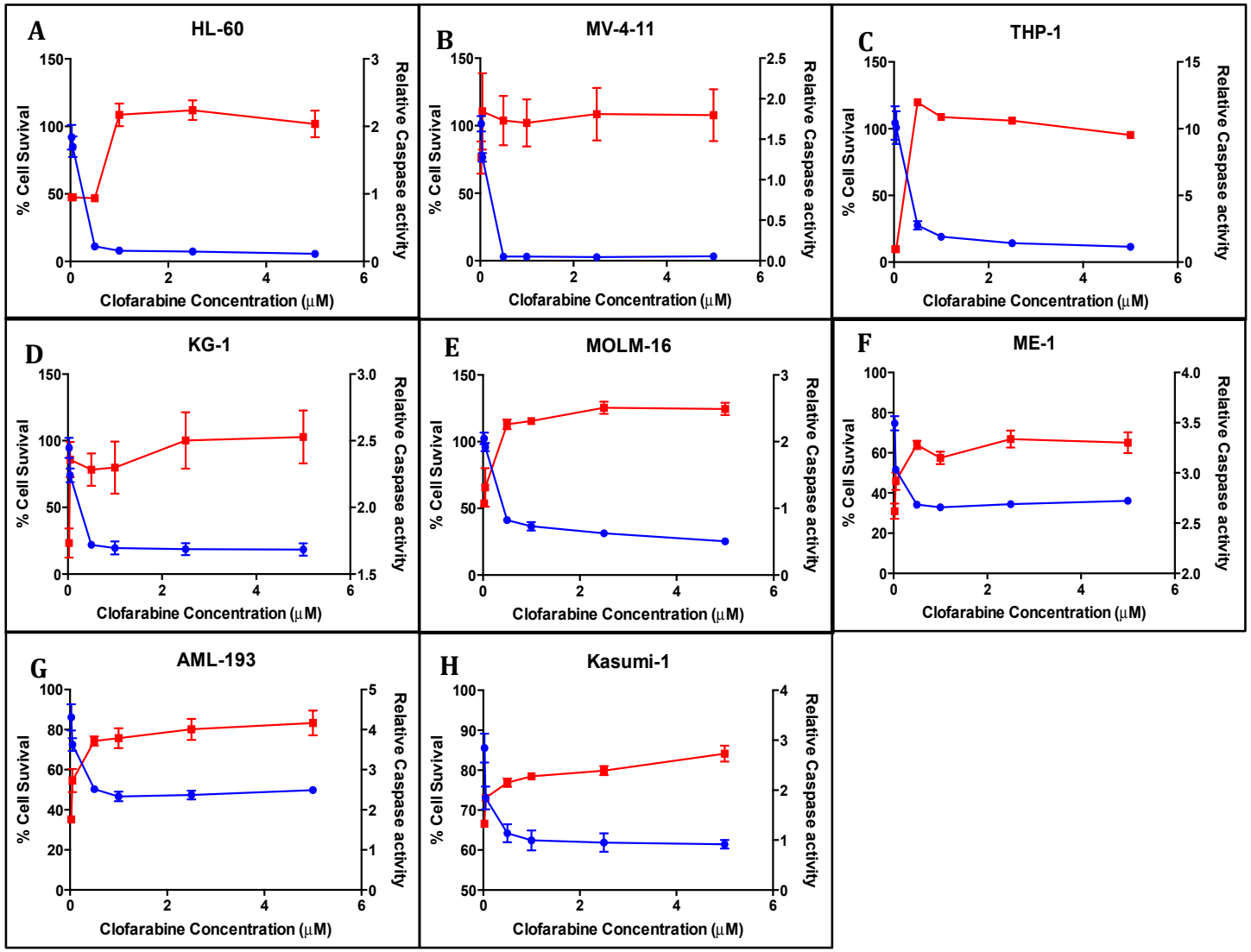

A
HL-60
% Cell Suvival
Relative Caspase activity
Clofarabine Concentration (μM)
B
MV-4-11
% Cell Suvival
Relative Caspase activity
Clofarabine Concentration (μM)
C
THP-1
% Cell Suvival
Relative Caspase activity
Clofarabine Concentration (μM)
D
KG-1
% Cell Suvival
Relative Caspase activity
Clofarabine Concentration (μM)
E
MOLM-16
% Cell Suvival
Relative Caspase activity
Clofarabine Concentration (μM)
F
ME-1
% Cell Suvival
Relative Caspase activity
Clofarabine Concentration (μM)
G
AML-193
% Cell Suvival
Relative Caspase activity
Clofarabine Concentration (μM)
H
Kasumi-1
% Cell Suvival
Relative Caspase activity
Clofarabine Concentration (μM)

Figure 5.3: **Characterization of AML Cell lines for Clofarabine chemosensitivity.** Clofarabine-induced cytotoxicity (represented as percent cell survival, blue line) and cytarabine-induced apoptosis (represented as relative caspase-3/7 activity, red line) in (A) HL-60, (B) MV-4-11, (C) THP-1, (D) KG-1, (E) MOLM-16, (F) ME-1, (G) AML-193, (H) Kasumi-1.

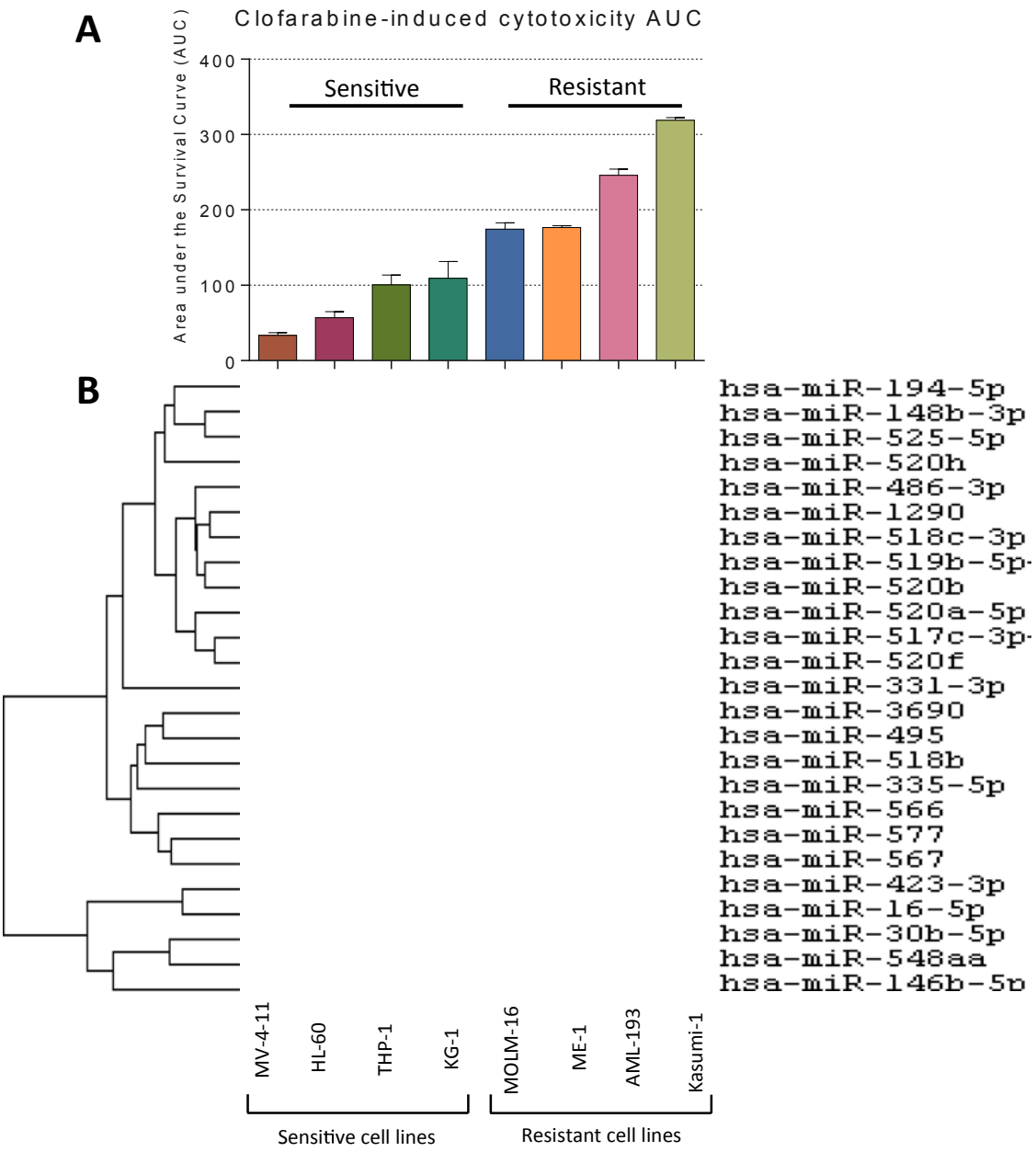


Figure 5.4: **Heatmap of microRNA expression in sensitive and resistant AML cell lines.** (A) Area under the survival curve (AUC) of 8 AML cell lines after 6 different concentrations of clofarabine. (B) Heatmap showing expression of top 25 differentially expressed microRNAs between sensitive and resistant AML cell lines.

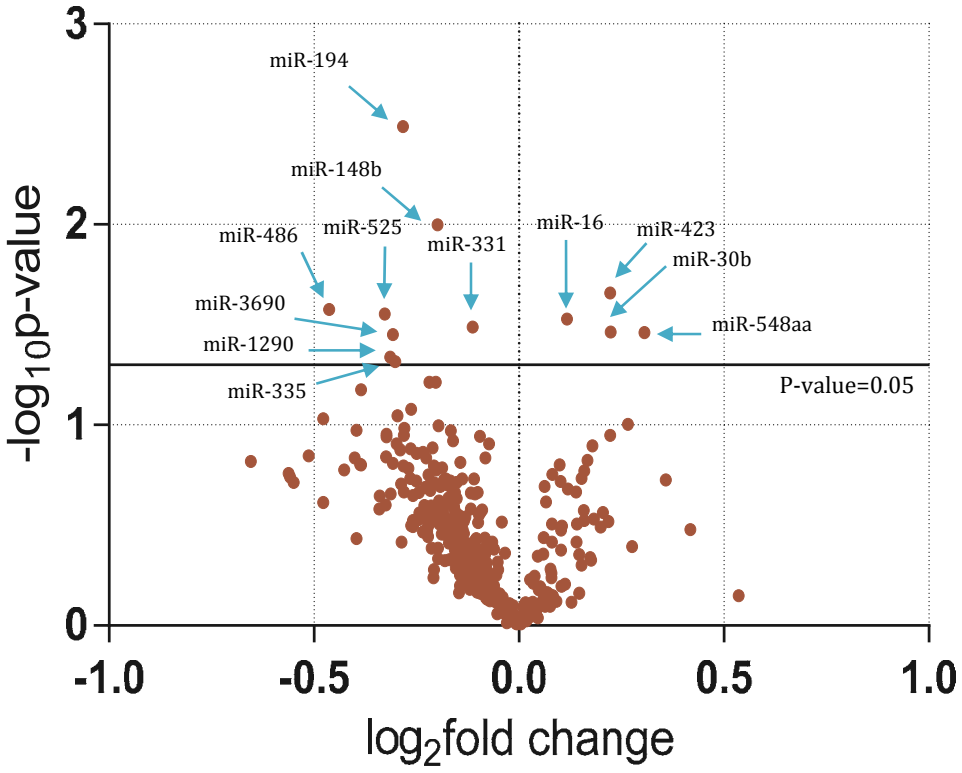


Figure 5.5: Differentially expressed microRNAs between sensitive and resistant cell lines to clofarabine

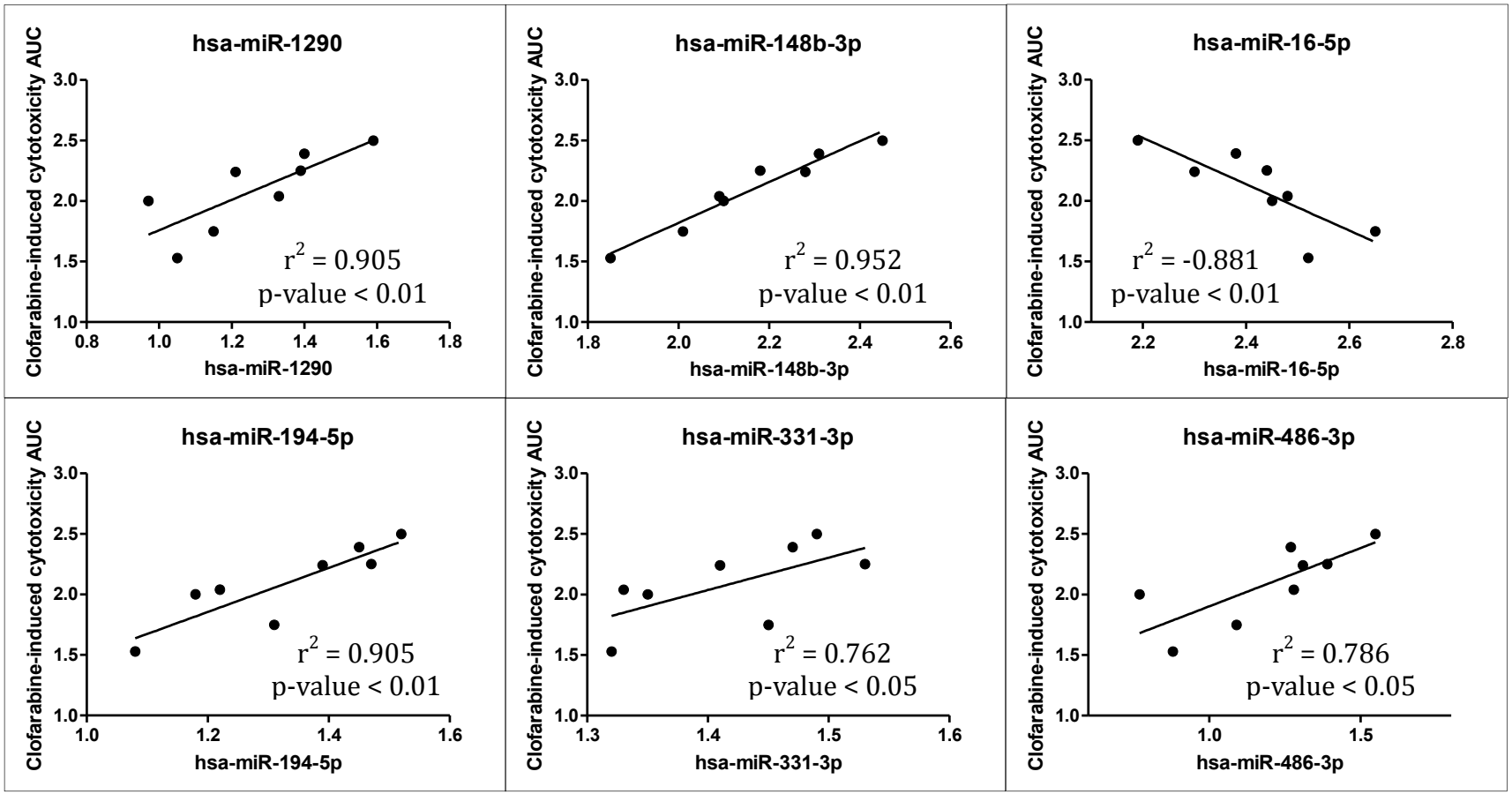


Figure 5.6: Correlation of clofarabine-induced cytotoxicity AUC and microRNA expression for the microRNAs, which were also differentially expressed microRNAs between sensitive and resistant cell lines to clofarabine.

Table 5.1: Characterization of AML Cell lines for Clofarabine Chemosensitivity

Cell Lines	Cytogenetics /Molecular Abnormality	Clofarabine-induced Cytotoxicity AUC (± SD)	Clofarabine-induced Apoptosis AUC (± SD)
MV-4-11	FLT3 ITD mutation, t(4;11)(q21;q23) → MLL-AF4 fusion gene	33.66 (±4)	8.8 (±1.6)
HL-60	CDKN2A, NRAS, TP53 mutant genes	56.84 (±8)	9.9 (±0.6)
THP-1	t(9;11)(p21;q23) → MLL-AF9 fusion gene; CDKN2A, KDM6A, NRAS mutant genes	100.58 (±13)	49.9 (±0.3)
KG-1	NRAS mutation, P53 mutation, RB1 rearrangement	109.10 (±22)	12.1 (±1.0)
MOLM-16	t(6;8)(q21;q24.3) and t(9;18)(q13;q21)	174.55 (±8)	11.8 (±0.4)
ME-1	inv(16)(p13q22) → CBFB-MYH11 fusion gene	176.40 (±2)	16.2 (±0.4)
AML-193	+der(17)t(17;17)(p13.1;q21.3)	245.73 (±9)	19.4 (±1.2)
Kasumi-1	t(8;21)(q22;q22) → RUNX1/AML1-RUNX1T1/ETO fusion gene; TP53 mutant gene	318.85 (±4)	12.1 (±0.9)

Table 5.2: MicroRNAs significantly associated with clofarabine-induced cytotoxicity AUC and clofarabine-induced apoptosis AUC

MicroRNAs	Spearman r	p-value
Clofarabine-induced cell viability AUC		
hsa-miR-1290	0.9048	0.0046
hsa-miR-148b-3p	0.9524	0.0011
hsa-miR-16-5p	-0.8810	0.0072
hsa-miR-194-5p	0.9048	0.0046
hsa-miR-22-3p	0.8333	0.0154
hsa-miR-331-3p	0.7619	0.0368
hsa-miR-486-3p	0.7857	0.0279
hsa-miR-500a-5p+hsa-miR-501-5p	0.8810	0.0072
hsa-miR-517a-3p	0.8095	0.0218
hsa-miR-517c-3p+hsa-miR-519a-3p	0.8571	0.0107
hsa-miR-518b	0.9048	0.0046
hsa-miR-518e-3p	0.8095	0.0218
hsa-miR-519b-3p	0.7381	0.0458
hsa-miR-519b-5p+hsa-miR-519c-5p	0.7619	0.0368
hsa-miR-520a-5p	0.9762	0.0004
hsa-miR-520b	0.8333	0.0154
hsa-miR-520c-3p	0.7619	0.0368
hsa-miR-520f	0.8571	0.0107
hsa-miR-520h	0.7857	0.0279
hsa-miR-522-3p	0.7619	0.0368
hsa-miR-525-5p	0.9524	0.0011
hsa-miR-526a+hsa-miR-520c-5p+hsa-miR-518d-5p	0.9048	0.0046
Clofarabine-induced Apoptosis AUC		
hsa-miR-27b-3p	-0.6988	0.0495
hsa-miR-301a-3p	-0.8503	0.0086
hsa-miR-3196	0.8144	0.0180

CHAPTER VI

Summary and Discussion

Acute myeloid leukemia (AML) is a hematological malignancy and is the most common forms of acute leukemia in adults with an incidence rate of 4.0501 cases per 100,000 patients. AML also has a highest mortality rate of 2.85 cases per 100,000 patients as compared to other leukemias and is associated with low 5-year relative survival rates (24.9% overall). Apart from low survival, one of the major concerns with AML is treatment related mortality and resistance to chemotherapy [1, 2]. Various prognostic factors such as cytogenetics, molecular abnormalities, and minimal residual disease mainly predict resistance to chemotherapy. Cytogenetic abnormalities have been strong predictors of the clinical outcomes in both pediatric and adult AML patients. However, despite using cytogenetics, around 20 to 45% patients do not achieve complete remission (CR) with standard induction chemotherapy with cytarabine (commonly used nucleoside analog) and the majority of the patients who achieve CR are known to relapse [3-5].

One of the major reasons for failure of cytarabine-based therapy is due to failure to achieve adequate intracellular active cytarabine triphosphate concentrations. The primary factors that regulate the intracellular triphosphate levels are the levels/activity of the nucleoside transporters, levels of the activating (kinases) and inactivating enzymes and the intracellular deoxynucleotide pools, which are regulated by ribonucleotide reductases. Factors that could affect the expression of these transporters and enzymes, could in turn potentially affect the levels of the active metabolite of nucleoside analogs.

Single nucleotide polymorphisms (SNPs) in the genes encoding the drug transporters, activating and inactivating enzymes as well as the pharmacodynamic (PD) targets of these drugs can influence the clinical outcome of the patients treated with these

nucleoside analogs. Studies have shown that variability in treatment outcome in AML patients treated with cytarabine correlated with DCK mRNA expression [6]. The regulatory SNPs (−360 C>G and −201 C>T) in the promoter of DCK were found to be associated with the mRNA expression of DCK [7]. Sequencing the DCK promoter and coding exons identified three nonsynonymous SNPs (Ile24Val, Ala119Gly and Pro122Ser), the variant forms of which demonstrated reduced DCK activity [8]. A SNP in the 3' untranslated region (UTR) of DCK (rs4643786) was found to be associated with DCK mRNA expression in both European and African ancestry. Additionally, SNPs in CDA, RRM2 and hENT1 have also been found to predict the clinical outcome/cytarabine triphosphate levels in AML patients. However, apart from SNPs, we were also interested in exploring microRNAs as predictors of response to cytarabine in AML patients. MicroRNAs (miRs) are endogenous, single stranded, small noncoding RNAs, which are about 22 nucleotides in length that regulate gene expression by binding to specific mRNA target sequences and promoting their degradation or translational repression. MicroRNAs have also been implicated to play an important biological functional role in the development of cancer.

Recently, various *in vitro* studies have also sought to identify the impact of microRNAs on the chemosensitivity to anticancer agents in various cancers. However, the data from these studies has been contradictory and disease dependent. Specifically, it was found that increasing the levels of miR-21 in A549 cell line leads to increased sensitivity to cytarabine, which was opposite to what was observed with other anticancer agents, where decreased expression of miR-21 resulted in increased sensitivity of these agents, in this as

well as other studies [9-11]. However, another study showed that inhibiting the expression of endogenous miR-21 enhanced the sensitivity of leukemic cell line (HL-60) to cytarabine, primarily by increasing apoptosis. However, despite the extensive knowledge regarding the role of microRNAs as regulators of various cellular processes in cancer (prognostic markers for AML) and influencing changes in chemosensitivity of anti-cancer agents in *in vitro* systems; there have been very few studies that have systematically evaluated the role of microRNAs as predictors of chemosensitivity to nucleoside analogs in AML patients. Thus, the aim of this thesis was to identify microRNA/microRNA-signature using *in vitro* systems and clinical data that can be used as a tool for predicting clinical outcome in AML patients. Additionally, we sought to mechanistically determine if these microRNAs were influencing the chemosensitivity of cytarabine by modulating the genes involved in intracellular activation of cytarabine.

We used an innovative approach to identify microRNAs predictive of cytarabine chemosensitivity using eight AML cell lines and further testing these microRNAs in an integrative analysis to identify specific miRNA-target mRNA pairs that were predictive of overall survival (OS) in AML. Briefly, we evaluated 412 microRNAs for association with cytarabine chemosensitivity in AML cell lines, of these 20 microRNAs were evaluated for their association with OS in 200 AML patients from TCGA database. Our stepwise-integrated analyses (step 1- microRNA-target mRNA that were significantly correlated in AML patients; step -2 mRNAs from step 1 with significant association with OS) identified 23 unique miRNA-mRNA pairs predictive of OS in AML patients. As expected HOX genes (HOXA9, HOXB7 and HOXA10) were identified to be regulated

by miRs as well as predictive of worse OS. Additionally, miR107-Myb, miR-378-granzyme B involved in granzyme signaling and miR10a-MAP4K4 were identified to be predictive of outcome through integrated analysis. Although additional functional validations to establish clinical/pharmacologic importance of microRNA-mRNA pairs are needed, our results from RNA EMSA confirmed binding of miR-10a, miR-378 and miR-107 with their target genes GALNT1, GZMB and MYB respectively. Integration of pathogenic and pharmacologically significant microRNAs and microRNA-mRNA relationships identified in our study opens up opportunities for development of targeted/microRNA-directed therapies.

Next, we sought to understand the potential mechanism by which microRNAs can influence the chemosensitivity to cytarabine. One of the primary mechanisms of resistance to nucleoside analogs is insufficient intracellular concentration of the active triphosphate metabolite. This insufficient triphosphate levels could be due to inefficient cellular uptake of the drug, reduced levels of the activating enzyme, increased levels of inactivating enzymes and/or due to increased levels of endogenous deoxynucleotide (dNTP) pool [12-15]. MicroRNAs have been shown to regulate the expression of various drug-metabolizing enzymes like CYP3A4 etc., drug transporters like BCRP and various drug targets [16-18]. However, there have not been any studies, which have comprehensively evaluated the effect of microRNAs on the important genes involved in the transport, activation and inactivation genes of nucleoside analogs. In our study we identified ~ 30 microRNAs which were significantly correlated with mRNA expression of various cytarabine pathway genes. Of the microRNAs identified in *in vitro* study, 5

microRNAs were also found to correlate with the gene expression in patients. Using *in vitro* and bioinformatic analysis we were able to show that the regulation of the genes was via binding to the 3'-UTR of the target genes. Future studies to understand the clinical impact of the microRNA mediated changes in gene expression on activity would involve correlating the *in vivo* microRNA expression with the plasma concentrations of cytarabine and/or cytarabine triphosphate.

Apart from microRNAs, we also investigated if treatment with cytarabine results in emergence of drug resistance due to changes in gene expression profile. In chapter IVA, we determined the gene expression in patients prior to and post treatment with cytarabine. Using PROMISE analysis, we identified more than 50 genes that were either associated with detrimental or beneficial response in AML patients treated with cytarabine. Using siRNA knockdown technique we were able to mechanistically validate the gene expression changes that were correlated with clinical outcome in the AML patients. This, to our knowledge is the first study that has identified drug-induced changes in gene expression of various adaptive processes that can contribute to development of resistance. The results highlight genes relevant to cytarabine resistance and support the concept of targeting genes modulated by cytarabine exposure as a means of improving response. Future in depth studies will help in understanding the interplay of these genes/pathways to better understand mechanisms of cytarabine resistance in AML. Importantly, novel agents directed at these targets may serve as potential therapeutics to improve clinical outcomes. In the next part of the chapter (chapter IVB) we sought to determine the effect of cytarabine treatment on changes in gene expression as well as microRNA expression.

In this study, we report the effect of cytarabine treatment at two different concentrations on the changes in microRNA expression in AML cell lines. We also investigated the effect of cytarabine treatment on the expression of pathway genes, in order to understand if the drug treatment itself can have an impact on its activation or inactivation pathway Using clinical data and mechanistic studies we were able to identify microRNA and drug induced changes in gene expression as being potential mechanisms of resistance to cytarabine in AML patients. However, apart from cytarabine, other second generation nucleoside analogs with favorable PK/PD properties are also being investigated as potential second generation drugs as treatment options in AML patients. Clofarabine is one such nucleoside analog that is currently being investigated in various clinical trials. Thus, in chapter V we performed *in vitro* studies to identify microRNAs that could predict chemosensitivity to clofarabine. Since we had observed good *in vitro*/*in vivo* correlation with cytarabine in AML cells lines and AML patients, and there are differences in PK pathway of clofarabine, we believe, that an investigational study using same *in vitro* system as was used for cytarabine (AML cell lines) would provide microRNA signature that is predictive of chemosensitivity to clofarabine. This microRNA signature can then be validated using clinical data from the ongoing clinical trials with clofarabine. Similar to as was done for cytarabine; we correlated the global microRNA expression profile in AML cells lines with the clofarabine chemosensitivity. We identified 25 unique microRNAs associated with clofarabine chemosensitivity, with miR-515 cluster significantly associated with clofarabine cytotoxicity. We also identified 8 microRNAs predictive of clofarabine sensitivity and 4 microRNAs to be predictive of

clofarabine resistance in AML cell lines. To our knowledge this is the first *in vitro* study that has identified microRNA signature that is predictive of clofarabine chemosensitivity. Additional functional validation and mechanistic studies are warranted to elucidate the role of these microRNAs in clofarabine response. Also validating these findings in AML patients would strengthen the significance of these microRNAs and help in better understanding the factors contributing to the response of these chemotherapeutic agents. In conclusion, over the last decade there have been significant efforts to identify genetic markers that can be used to explain the variability in response and predict clinical outcome in AML patients treated with cytarabine. However, there is still a clear need to identify additional mechanistic biomarkers that can help optimize the cytarabine dosing regimen that would help improve the clinical outcome and reduce the toxicities. Based on our work in this thesis we believe that microRNA signature can be a promising prognostic and predictive tool that can be used to individualize treatment therapy with cytarabine in AML patients.

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