Bioinformatic analysis of the effects and mechanisms of decitabine and cytarabine on acute myeloid leukemia

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Abstract. Acute myeloid leukemia (AML) is a frequently occurring malignant disease of the blood and may result from a variety of genetic disorders. The present study aimed to identify the underlying mechanisms associated with the therapeutic effects of decitabine and cytarabine on AML, using microarray analysis. The microarray datasets GSE40442 and GSE40870 were downloaded from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) and differentially methylated sites were identified in AML cells treated with decitabine compared with those treated with cytarabine via the Linear Models for Microarray Data package, following data pre-processing. Gene Ontology (GO) analysis of DEGs was performed using the Database for Annotation, Visualization and Integrated Analysis Discovery. Genes corresponding to the differentially methylated sites were obtained using the annotation package of the methylation microarray platform. The overlapping genes were identified, which exhibited the opposite variation trend between gene expression and DNA methylation. Important transcription factor (TF)-gene pairs were screened out, and a regulated network subsequently constructed. A total of 190 DEGs and 540 differentially methylated sites were identified in AML cells treated with decitabine compared with those treated with cytarabine. A total of 36 GO terms of DEGs were enriched, including nucleosomes, protein-DNA complexes and the nucleosome assembly. The 540 differentially methylated sites were located on 240 genes, including the acid-repeat containing protein (*ACRC*) gene that was additionally differentially expressed. In addition, 60 TF pairs and overlapped methylated sites, and 140 TF-pairs and DEGs were screened out. The regulated network included 68 nodes and 140 TF-gene pairs. The present study identified various genes including *ACRC* and proliferating cell nuclear antigen, in addition to various TFs, including TATA-box binding protein associated factor 1 and CCCTC-binding factor, which may be potential therapeutic targets of AML.

Introduction

Acute myeloid leukemia (AML) is one of the most frequently occurring malignant diseases of the blood and patients of all ages may present with symptoms. It has previously been reported that AML in children accounts for 25% of pediatric leukemia cases and affects ~180 patients annually in Japan (1). A total of 19,000 cases of AML are diagnosed each year, with ~10,000 of these in the United States (2). Outcomes have improved in younger patients, with a 40-50% 5-year overall survival rate (3). However, the majority of AML cases occur in adults, and in these cases the mortality rate remains high. It has been demonstrated that only 10-20% of patients aged >60 years survive to 5 years; 80% of patients are incurable as a result of primary refractoriness, relapse or treatment-associated mortality (4,5). AML has several subtypes and treatment and prognosis varies among them. AML is treated traditionally with chemotherapy and recent genetic research has provided more personalized treatment options. Clinicians can now predict which drug or drugs may work best for a particular person, and how long that person is likely to survive. Furthermore, numerous studies have reported that various genetic abnormalities in the following genes: Nucleophosmin 1, runt related transcription factor 1, Tet methylcytosine dioxygenase 2 and isocitrate dehydrogenase [NADP(+)] 1 cytosolic, are associated with the occurrence, progression and recurrence of AML and may therefore be used to predict prognosis and guide future therapeutic research (6-9). Döhner *et al* (4) summarized the frequency and clinical significance of various important mutated genes. The primary first line therapeutic for the treatment of AML is combined chemotherapy with anthracycline

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and cytarabine (10). Further therapeutic options include the hypomethylating agents decitabine and azacitidine) low-dose cytarabine, investigational agents, and supportive care with hydroxyurea and transfusions (11). Decitabine is a deoxynucleoside analogue of cytidine that selectively inhibits DNA methyltransferases. It is considered an effective and well-tolerated alternative treatment to cytarabine or supportive care in older patients with AML (12). To improve the efficacy and structure of decitabine, the present study examined the mechanism underlying the effects of decitabine and cytarabine on AML, via microarray analysis. Although some progress has been made in targeted therapy of AML, the diagnosis and treatment of it remain challenging. The present study identified additional biomarkers associated with the therapeutic effects of drugs, in order to explore the corresponding mechanisms.

Materials and methods

Microarray data. The microarray datasets GSE40442 (13) and GSE40870 (13) were downloaded from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih. gov/geo/). The expression profile of GSE40442 contained 67 primary AML samples cultured with medium only for 1 day, followed by 3 days treatment with decitabine (the case group; n=17), cytarabine (the control group; n=16), dimethyl sulfoxide (DMSO; n=17) or untreated (n=17). These data were identified via the GPL5188 [HuEx-1_0-st] Affymetrix Human Exon 1.0 ST Array [probe set (exon) version] platform. The GSE40870 profile presented the methylation data of AML cell samples treated with decitabine (the case group; n=16), cytarabine (the control group; n=16) or DMSO (n=16). Detection of the methylation data was performed via GPL13534 Illumina HumanMethylation450 BeadChip (HumanMethylation 450_15017482).

Data preprocessing. To create the expression profile, the original data were converted into a recognizable format in *R*, and the affy (14) package (bioconductor.org/ packages/release/bioc/html/affy.html) was used for background correction and normalization, followed by conversion from the probe symbol to the gene symbol with the biomaRt (15) package of *R* (bioconductor.org/pack-ages/release/bioc/html/biomaRt.html). The β-value of every methylated site in all samples was extracted via GenomeStudio software version 2.0 (Illumina, Inc., San Diego, CA, USA) to create a methylation profile.

Identification of differentially expressed genes (DEGs) and differentially methylated sites. For GSE40442, the Linear Models for Microarray Data (16) package of *R* (bioconductor. org/packages/release/bioc/html/limma.html) was used to identify the DEGs in AML cells treated with decitabine compared with those treated with cytarabine. The DEGs were identified according to the criteria P<0.05 and log(fold-change)>0.5. The heatmap of DEGs in every sample of the control and the case group was constructed. For GSE40870, the differentially methylated sites were identified in AML cells treated with decitabine compared with cytarabine via the Illumina

Methylation Analyzer (17) package of *R* (ima.r-forge.r-project. org/), and were screened out with the criteria P<0.05 and log(fold-change)>0.2.

Functional enrichment analysis. Gene Ontology (GO) enrichment analysis of DEGs was performed via the Database for Annotation, Visualization and Integrated Discovery (david. abcc.ncifcrf.gov/) (18) with the threshold of P<0.05.

Screening of important genes and methylated sites. Genes corresponding to the differentially methylated sites were obtained by the annotation package of the methylation microarray platform. The genes that exhibited an overlap compared with DEGs were selected, and those that exhibited the opposite trend in the methylation variation compared with their expression were screened out.

Identification and analysis of important transcription factor (TF)-gene pairs and establishment of TF-gene regulated network. Methylation in the gene promoter region may affect the binding of TFs to genes and result in the variation of gene expression. Firstly, chromosomal locations of the methylation sites were identified using the annotation package of the methylation microarray platform. Following this, chromosomal locations of all the known and predicted TF binding sites were downloaded from the University of California Santa Cruz (UCSC) database (19) (genome.ucsc. edu/). The methylation sites were considered to affect the binding of TFs and genes when the chromosomal location of the methylation sites overlapped with the region of the TF binding site. Furthermore, all the known and predicted TF-gene pairs were downloaded from UCSC and the TF-gene pairs were screened out. The TF-gene regulated network was established via Cytoscape version 3.11 (www. cytoscape.org/).

Results

DEGs and differentially methylated sites. A total of 190 DEGs (102 up- and 88 downregulated) and 540 differentially methylated sites were identified in AML cells treated with decitabine compared with cytarabine, and all the identified differentially methylated sites were hypomethylated. The top 30 DEGs and the top 30 differentially methylated sites are presented in Tables I and II, respectively, and the heatmap of DEGs is presented in Fig. 1.

Enriched GO terms of the DEGs. A total of 36 enriched GO terms of DEGs, including nucleosome, protein-DNA complex, and nucleosome, chromatin and protein-DNA complex assemblies, were obtained and are presented in Table III.

Important genes and methylated sites. A total of 240 genes were screened, in which 540 differentially methylated sites were identified. These 240 genes were compared with the 190 DEGs, and the acid-repeat containing protein (*ACRC*) exhibited an overlap. Furthermore, *ACRC* corresponded to the methylated site of cg26924445 and demonstrated the opposite trend in the methylation variation compared with gene expression.

Table I. Top 30 differentially expressed genes in acute myeloid leukemia cells treated with decitabine compared with those treated with cytarabine.

Gene	Log(fold-change)	P-value
PNMA5	1.008734	4.61×10^{-7}
COL14A1	0.981479	7.39×10^{-6}
LINC01344	0.688924	1.12×10^{-5}
PPP1R27	0.991804	1.52×10^{-5}
ACRC	0.794564	1.99×10^{-5}
TKTL1	1.047284	2.91×10^{-5}
DAZL	0.504424	0.000131
RBMY3AP	-0.50665	0.000302
MIR675	0.598142	0.000474
MYBL2	-0.62118	0.000611
BNIP3P9	0.505733	0.00071
HIST1H1C	0.518146	0.000896
TK1	-0.63139	0.000925
HIST1H1E	0.612766	0.001018
PCNA	-0.52607	0.001235
TRAJ13	-0.96523	0.001403
RN7SKP60	0.640781	0.001425
CDKN1A	-0.61749	0.0015
HMGN5	0.575089	0.00238
NFE4	1.069562	0.002412
YPEL5P1	0.869379	0.002471
FAM111B	-0.86319	0.002552
HIGD1AP8	0.692903	0.002888
OR2L3	0.913212	0.003109
OR52P2P	-1.04122	0.003232
MDM2	-0.54666	0.003275
GACAT2	0.692088	0.003317
CCT4P2	-0.72233	0.003713
HIST1H1T	0.761500	0.003745
TMEM261P1	-0.52041	0.003776

Table II. Top 30 differentially methylated sites in AML cells treated with decitabine compared with those treated with cytarabine.

Methylation	Log(fold-change)	P-value
cg22040989	-0.46477	7.71×10^{-27}
cg19098118	-0.32461	3.17×10^{-22}
cg14063817	-0.34161	1.51×10^{-19}
cg17631454	-0.33468	5.13×10^{-19}
cg02597199	-0.32804	1.82×10^{-18}
cg08071595	-0.35059	3.15×10^{-18}
cg27576136	-0.20948	1.73×10^{-17}
cg09374462	-0.25800	1.77×10^{-17}
cg12442125	-0.35154	4.70×10^{-17}
cg05592278	-0.31725	4.70×10^{-17}
cg27052900	-0.24157	4.70×10^{-17}
cg22802167	-0.26297	5.69×10^{-17}
cg08550094	-0.35068	6.61×10^{-17}
cg21486341	-0.20684	6.79×10^{-17}
cg08411833	-0.27190	8.08×10^{-17}
cg17806847	-0.25718	8.08×10^{-17}
cg03865944	-0.20673	9.44×10^{-17}
cg03611733	-0.21888	9.80×10^{-17}
cg12091641	-0.31272	1.43×10^{-16}
cg17338368	-0.24704	2.00×10^{-16}
cg23641672	-0.21018	2.87×10^{-16}
cg23836413	-0.20551	2.87×10^{-16}
cg05073880	-0.22513	3.63×10^{-16}
cg12866103	-0.29918	5.32×10^{-16}
cg03282689	-0.25659	6.36×10^{-16}
cg07042346	-0.24842	8.04×10^{-16}
cg09014775	-0.21864	8.09×10^{-16}
cg05303739	-0.20861	1.05×10^{-15}
cg11017535	-0.24066	1.51×10^{-15}
cg13987334	-0.23196	1.51×10^{-15}

Important TF-gene pairs and the TF-gene regulated network. A total of 60 TF-gene pairs and overlapped methylated sites were screened out, including cg22475974-SET domain bifurcated (*SETDB)1*, cg14063817-estrogen receptor (*ER*)α*A*, cg22475974-*ER*α*A*, cg05835309-*SETDB1* and cg00334293-signal transducer and activator of transcription-3. In addition, 140 pairs of TFs and DEGs were identified, including CCAAT/enhancer binding protein β (*CEBPB*)-cysteine rich secretory protein 3, *CEBPB*-C-X-C motif chemokine ligand 2, *CEBPB*-fanconi anemia complementation group I, *CEBPB*-histone cluster 1 H1 family member C and *CEBPB*-microRNA 378e. In addition, the 60 pairs of TFs and overlapped methylated sites contained 20 TFs and 51 methylated sites. The TF-gene regulated network was established according to the 140 TF-gene pairs (Fig. 2). A total of 68 nodes and 140 pairs were involved in the regulated network. Furthermore, the 68 nodes contained 18 TFs (presented as

triangles) and 50 genes (presented as circles). The top 20 nodes according to connections with other nodes in the network are presented in Table IV.

Discussion

Decitabine (Dacogen®; 5-aza-2'-deoxycytidine) has been extensively used for the treatment of AML as an inhibitor of DNA methylation, which triggers demethylation leading to consecutive reactivation of epigenetically silenced tumor suppressor genes (20). When administered at low doses, decitabine may reduce genomic DNA methylation as a consequence of irreversible binding to DNA methyltransferases following incorporation into newly synthesized DNA (21). Cytarabine inhibits DNA synthesis by suppressing DNA polymerase activity; however, it additionally inhibits the elongation of the polynucleotide chain and interferes with
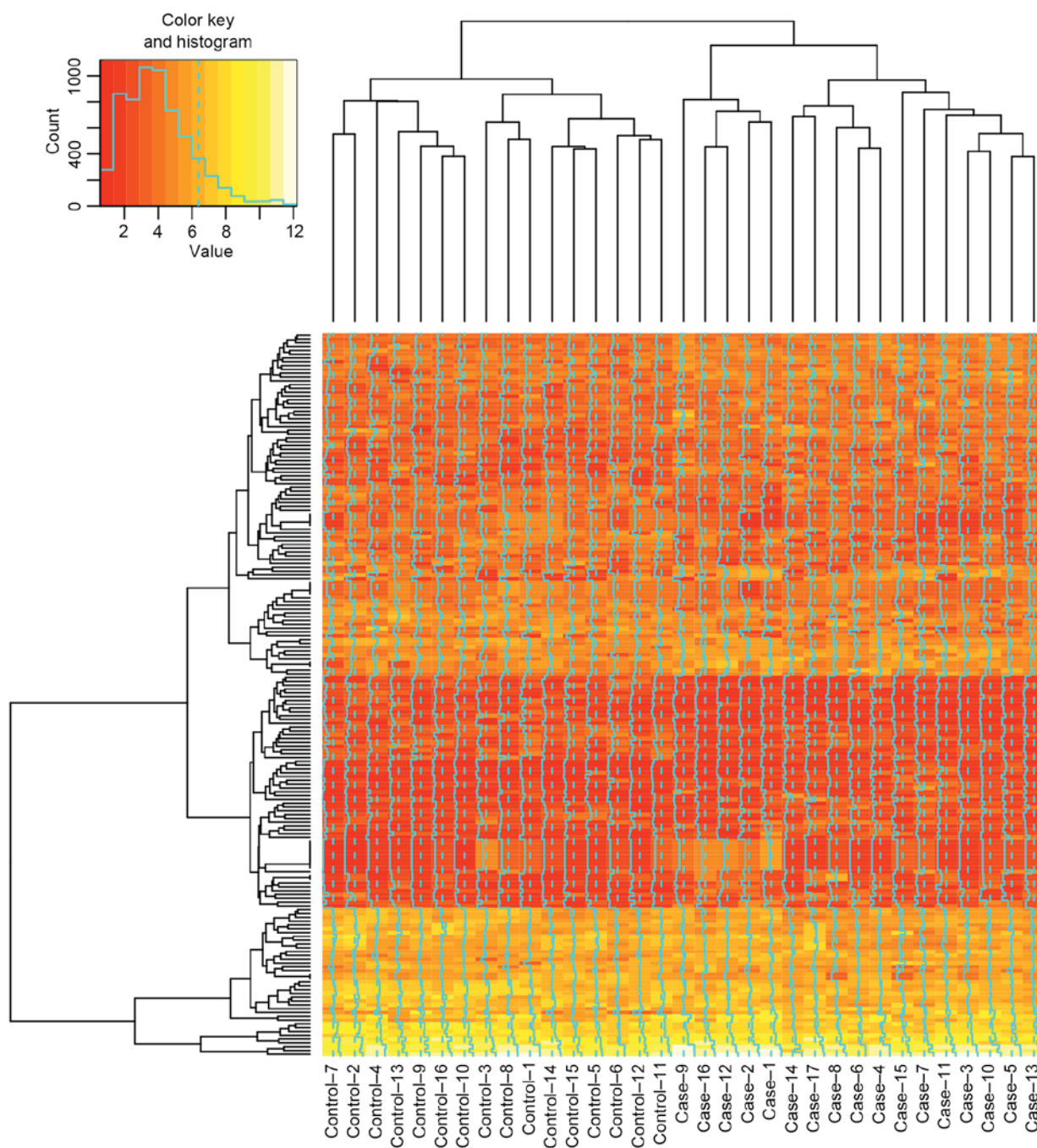
Figure 1. Heatmap of differentially expressed genes in all samples of control and case groups. A total of 16 AML cell samples treated with cytarabin served as the control group. A total of 16 AML cell samples treated with decitabine served as the case group. AML, acute myeloid leukemia.

the physiological function of DNA, which is important for the treatment of hematological malignancies (22,23). In the present study, all the identified differentially methylated sites were hypomethylated in the primary AML samples treated with low-dose decitabine compared with cytarabine, which is consistent with differing underlying mechanisms of decitabine and cytarabine in AML. The AML cell samples treated with decitabine differed from those treated with cytarabine in the heatmap of DEGs.

In the present study, a total of 36 GO terms enriched in DEGs were obtained. They were primarily associated with the combination of protein and DNA, (protein-DNA complex and protein-DNA complex assembly), chromosome conformation (chromatin assembly or disassembly, chromosomal part and chromatin organization), and biological processes associated with the assembly of macromolecular complexes (nucleosome assembly, protein-DNA complex assembly, macromolecular complex assembly and cellular macromolecular complex assembly). It has previously been demonstrated that DNA methylation is important in the biological processes of genomic imprinting, X-chromosome inactivation, suppression of transposable elements and carcinogenesis (24-28). DNA methylation is considered a potent epigenetic modification and may inhibit TF recruitment, resulting in suppression of transcription (24,29), and closely associates with health and disease in humans (30,31). Furthermore, it has been

Table III. Enriched GO terms of differentially expressed genes.

Category	GO ID	GO name	P-value
CC	0000786	Nucleosome	1.03×10^{-7}
CC	0032993	Protein-DNA complex	6.69×10^{-7}
BP	0006334	Nucleosome assembly	8.70×10^{-7}
BP	0031497	Chromatin assembly	1.07×10^{-6}
BP	0065004	Protein-DNA complex assembly	1.40×10^{-6}
BP	0034728	Nucleosome organization	1.59×10^{-6}
BP	0006323	DNA packaging	6.06×10^{-6}
CC	0000785	Chromatin	7.65×10^{-6}
BP	0006333	Chromatin assembly or disassembly	9.73×10^{-6}
CC	0005694	Chromosome	3.77×10^{-5}
CC	0044427	Chromosomal part	7.21×10^{-5}
BP	0016584	Nucleosome positioning	2.24×10^{-4}
BP	0065003	Macromolecular complex assembly	0.001008
BP	0034622	Cellular macromolecular complex assembly	0.001458
CC	0031012	Extracellular matrix	0.001562
BP	0043933	Macromolecular complex subunit organization	0.001593
BP	0034621	Cellular macromolecular complex subunit organization	0.002613
BP	0006259	DNA metabolic process	0.003411
BP	0006325	Chromatin organization	0.003467
CC	0005654	Nucleoplasm	0.004301
CC	0005578	Proteinaceous extracellular matrix	0.006181
BP	0006260	DNA replication	0.006457
CC	0044421	Extracellular region part	0.007453
BP	0051276	Chromosome organization	0.011355
MF	0003677	DNA binding	0.012103
BP	0006974	Response to DNA damage stimulus	0.015069
BP	0030162	Regulation of proteolysis	0.018269
BP	0033554	Cellular response to stress	0.022660
BP	0006281	DNA repair	0.024911
MF	0005125	Cytokine activity	0.024942
BP	0032026	Response to magnesium ion	0.030924
CC	0031981	Nuclear lumen	0.035330
BP	0046685	Response to arsenic	0.038507
CC	0000307	Cyclin-dependent protein kinase holoenzyme complex	0.039727
BP	0051726	Regulation of cell cycle	0.040353
MF	0004984	Olfactory receptor activity	0.049749

GO, Gene Ontology; CC, cellular component; BP, biological process; MF, molecular function.

previously reported that DNA methylation is an epigenetic activity that affects the structure of chromosomes, but not the sequence of genes (32-34). Therefore, the aforementioned data demonstrated that decitabine may affect AML via gene methylation.

Of the identified DEGs, *ACRC* was the only one to additionally contain a differentially methylated site, cg26924445, and demonstrated an opposite trend in methylation variation compared with expression. Nestheide *et al* (35) suggested that *ACRC* is an important biomarker of Ewing sarcoma and concludes that epigenetic dysregulation may contribute to the pathogenesis of angiosarcoma, via analysis of expression and methylation profiles. The results of the present study demonstrated that decitabine can alter the methylation status of cg26924445, and that as in their study, increasing expression of *ACRC* was conducive to treating AML. Therefore, it was suspected that decitabine might treat AML through altering the methylation status of cg26924445 in *ACRC*. The results of the present study revealed that the TATA-box binding protein associated factor 1 (TAF1) regulated the most genes or TFs in the TF-gene regulated network. Therefore, TAF1 may act as a critical TF for decitabine treatment of AML, and may be important in the differing underlying molecular mechanisms of decitabine and cytarabine. Ben Abdelali *et al* (36)
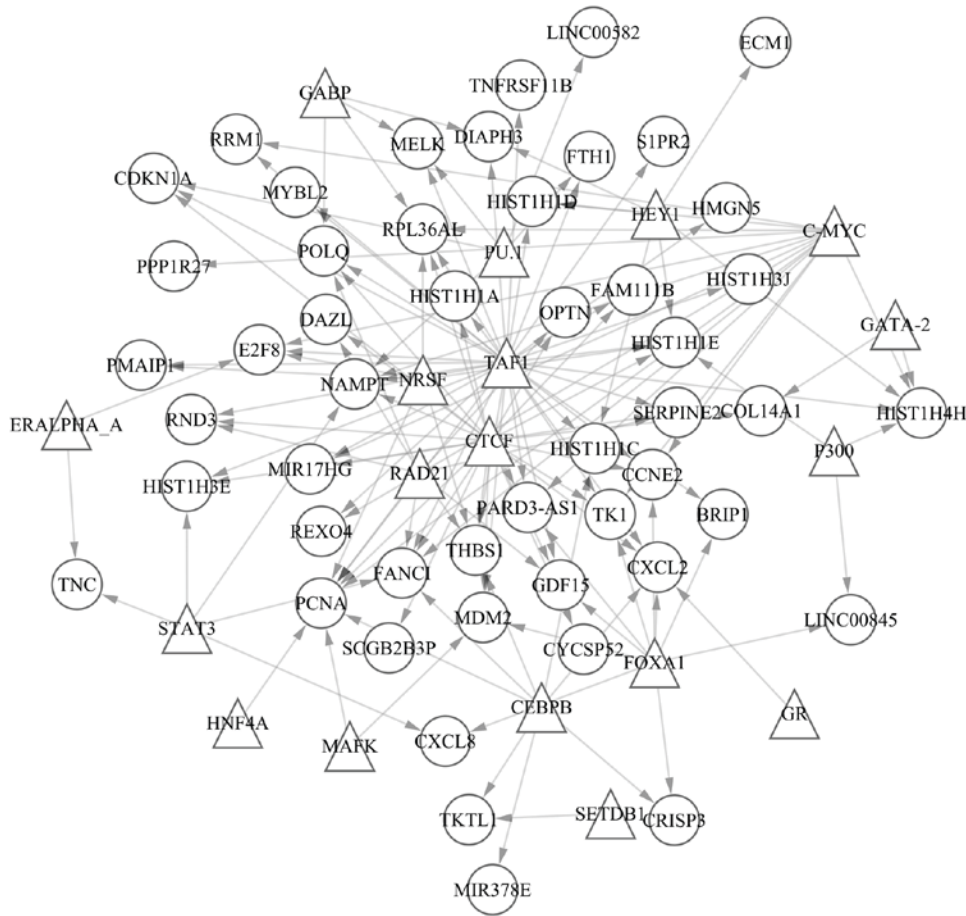
Figure 2. TF-gene regulated network. Triangles represent TF nodes; circles represent gene nodes. TF, transcription factor.

Table IV. Top 20 most significant nodes according to degree.

Node	Degree
TAF1	36
CTCF	25
C-MYC	14
FOXA1	10
RAD21	9
CEBPB	8
PCNA	8
HEY1	6
NRSF	6
FANCI	6
PU.1	5
STAT3	5
CXCL2	5
THBS1	5
HIST1H4H	5
NAMPT	5
RPL36AL	5
HIST1H1E	5
GABP	4
CCNE2	4

Degree, connection with other nodes.

reported that the SET-NUP214 (TAF1/CAN) fusion gene is an important influencing factor in the survival rate of AML. Therefore, TAF1 may be a potential novel target gene in decitabine-treated AML. The CCCTC-binding factor (CTCF) was another TF that regulated numerous genes or TFs. Manodoro *et al* (37) demonstrated that in AML, the methylation of CTCF binding sites may result in loss of imprinting at 14q32. Furthermore, the present study demonstrated that proliferating cell nuclear antigen (*PCNA)* was the gene regulated by the greatest number of TFs, and Buchi *et al* (38) reported that the expression of *PCNA* was altered in AML treated with decitabine or cytarabine.

In conclusion, the results of the present study suggested that decitabine suppresses the function of certain antitumor genes via methylation, in its role as a therapeutic agent for the treatment of AML, and that this underlying mechanism of action differs to that of cytarabine. The present study provides information regarding potential drug targets, which may improve the efficacy of decitabine in the treatment of AML.

Acknowledgements

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