Bioinformatics analysis of the network of histone H3 lysine 9 trimethylation in acute myeloid leukaemia

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Abstract. Changes in histone H3 lysine 9 trimethylation (H3K9me3) may be related to the development of drug-resistant acute myeloid leukaemia (AML); insights into the network of H3K9me3 may improve patient prognosis. Patient data were derived from the Gene Expression Omnibus (GEO) database and data from AML cells treated with chidamide, a novel benzamide chemical class of histone deacetylase inhibitor (HDACi), *in vitro* were derived from ChIP-seq. Patients and AML cell data were analysed using GEO2R, GOseq, KOBAS, the STRING database and Cytoscape 3.5.1. We identified several genes related to the upregulation or downregulation of H3K9me3 in AML patients; some of these genes were related to apoptosis, autophagy, and the pathway of cell longevity. AML cells treated with chidamide *in vitro* showed the same gene changes. The protein interactions in the network did not have significantly more interactions than expected, suggesting the need for more research to identify these interactions. One compelling result from the protein interaction study was that sirtuin 1 (SIRT1) may have an indirect interaction with lysine-specific demethylase 4A (KDM4A). These results help explain alterations of H3K9me3 in AML that may direct further studies aimed at improving patient prognosis. These results may also provide a basis for chidamide as a treatment strategy for AML patients in the future.

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Introduction

Acute myeloid leukaemia (AML) is one of the most common malignant clonal diseases of the circulatory system; it carries a high mortality rate (including a high treatment-related mortality rate of approximately 1.57/100,000 individuals per year in China) and a high recurrence rate (1). The prognosis of most AML patients is poor. The cure rate of AML patients (except for that of acute promyelocytic leukaemia) under 60 years of age is 35-40%, but this rate is only 5-15% in patients over 60 years of age in China. Strategies designed to improve prognosis and to improve these high rates are the foci of investigation among researchers.

Histone modification is an epigenetic event related to the prognosis of malignant haematologic diseases; histone deacetylase was recently found to be associated with the prognosis in lymphoma (2). A study reported that loss of function and deletions in zeste homolog 2 (EZH2) [a histone methyltransferase that is responsible for transcriptional repression of target genes by trimethylation of lysine 27 on histone H3 (H3K27me3)] are frequent in myeloid malignancies such as myelodysplastic syndromes (MDS), atypical chronic myelogenous leukaemia (CML), T cell acute lymphoblastic leukaemia (T-ALL) and myelofibrosis; these mutations are generally associated with poorer patient prognosis with reduced overall survival (OS) and event-free survival (3-9). Mixed-lineage leukaemia (MLL)-rearranged leukaemias have distinct clinical features and poor prognosis. The majority of MLL translocations result in oncogenic fusion proteins in which the native methyltransferase domain is replaced with sequences that interact with disruptor of telomeric silencing 1-like (DOT1L) directly or indirectly. MLL-rearranged leukaemia depends on aberrant histone H3 lysine 79 (H3K79) methylation by DOT1L (10,11). Another study showed that H3K9me3 deregulation in AML occurred preferentially as a decrease in histone H3 lysine 9 trimethylation (H3K9me3) levels at core promoter regions. When the H3K9me3 signature was combined with established clinical prognostic markers, it outperformed prognosis predictions based on clinical parameters alone (12). Taken together, these studies suggest that histone methylation is important for prognosis; nevertheless, there have been no studies of the pathways or regulatory mechanisms of H3K9me3 in AML cell lines.

Previous research from our team found that levels of H3K9me3 could be affected by chidamide, a novel benzamide chemical class of histone deacetylase inhibitor (HDACi), an agent that alters expression levels of sirtuin 1 (SIRT1) (a histone deacetylase), and enhances the cytotoxicity of drugs in AML cells (13). Therefore, we performed the present study to explore the pathway of H3K9me3 as well as the regulatory mechanisms of SIRT1 on H3K9me3. This study may also provide effective treatment strategies consequently improving the prognosis of AML patients.

Materials and methods

Samples and databases. A total of 108 primary AML samples and 36 control samples were selected from the Gene Expression Omnibus (GEO) database [GEO accession no. GSE20452 (12), last update, March 22, 2012]. In GSE20452, blasts from patients with AML were obtained at the time of diagnosis. Two batches of experiments were performed and analysed separately. One group of specimens contained AML samples (n=38) and the other contained AML samples (n=70), CD34$^+$ progenitor cells (n=21) and white blood cells (n=15) as controls. We used GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) to compare two groups of samples so as to identify genes that are differentially expressed across experimental conditions. GEO2R terms with corrected P-values <0.05 were considered significant, as were logFC of GEO2R terms >1.0.

Different peak analysis. Different peak analysis was based on the fold-enrichment of peaks in various experiments. A peak was defined as different when the odds ratio (OR) between two groups was more than 2. Using the same method, genes associated with different peaks were identified and subjected to Gene Ontology (GO) enrichment analysis. We used KEGG Orthology-Based Annotation System (KOBAS) 3.0 online (14) to test the statistical enrichment of peak-related genes in Kyoto Encyclopedia of Genes and Genomes (KEGG) (15-17) pathways. GO terms with corrected P-values <0.05 were considered significantly enriched by peak-related genes.

Analysis of protein interactions. The STRING (https://string-db.org/) database provides protein-protein interaction (PPI) information, including direct (physical) and indirect (functional) associations (18). Pathways from KEGG and the extended network were constructed for m1A regulators and related protein-coding genes signatures using Cytoscape 3.5.1.

Cell line. The AML cell line THP-1 was kindly donated by Professor Ravi Bhatia (City of Hope National Medical Center, Duarte, CA, USA). The THP-1 cell line was cultured in Iscove's modified Dulbecco's medium or RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% foetal bovine serum (FBS) at 37˚C in a humidified incubator containing 5% CO_2.

Drug treatment. Chidamide was kindly donated by Shenzhen Chipscreen Co. AML cells were treated for 24 h. For THP-1 cells, the dose of chidamide was 0.5 μM (20).

Chromatin immunoprecipitation sequencing (ChIP-seq). Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (13,21), and ChIP-seq was based on the Illumina Technology Sequencing platform (Illumina, Inc., USA). The single/paired-end method (13,21) was used to complete the ChIP-seq sequencing analysis of the THP-1 cell line. The antibody against histone was H3K9me3 (cat. #4260, RRID: AB_10828006; Cell Signaling Technology, Inc.).

Statistical analysis. The significances of differences were calculated using the moderated t-statistic (only available when two groups of samples were defined). P-values were adjusted for multiple testing. Genes with the smallest P-values were considered the most reliable. P<0.05 after adjustment was considered statistically significant. Log2-fold change between two experimental conditions (only available when two groups of samples were defined) was carried out. Moderated F-statistic combined the t-statistics was conducted for all pair-wise comparisons into an overall test of significance for that gene (only available when more than two groups of samples are defined).

Results

Differential expression of genes across experimental conditions between AML and control samples. When samples were collected from the GEO database (accession no. GSE20452), the differential expression of genes between AML samples and control samples was analysed using GEO2R. There were more than 2,000 genes that showed significant differential expression. The definition of the value in log(FC) for upregulation was >1.0, and the definition of the value in log(FC) for downregulation was <-1.0. According to the definition of upregulation or downregulation together with P<0.05, there were 147 genes related to alterations in H3K9me3 showing downregulation and 170 genes related to the change in H3K9me3 showing upregulation (data not shown).

Function and network analysis. The function of genes collected from GEO were further analysed using geometric mean of semantic similarities in biological processes (BPs), molecular functions (MFs), and cellular components (CCs), that were assessed using the GOSemSim package (22) by considering the GO topological structure in a more precise and unbiased manner. In BPs, there were 20 processes that showed significant changes (Fig. 1A). The main processes were related to metabolism, including cofactor metabolic process, cellular metabolic processes, and others. In MFs, there were only three significantly altered processes: Protein binding, binding, and molecular-function (Fig. 1B). In CCs, there were 20 processes that showed significant alterations, including intracellular and extracellular processes (Fig. 1C). The relationship of GO items in the regulation of apoptosis and autophagy are shown in Fig. 2A and B. These GO items were included in BPs, MFs, and CCs.

The related pathways were predicted using KOBAS 3.0 online. One of these pathways, the longevity pathway (Fig. 3), may related to the survival of leukaemia cells, including the PI3K/AKT pathway, the AMPK pathway, the FOXO
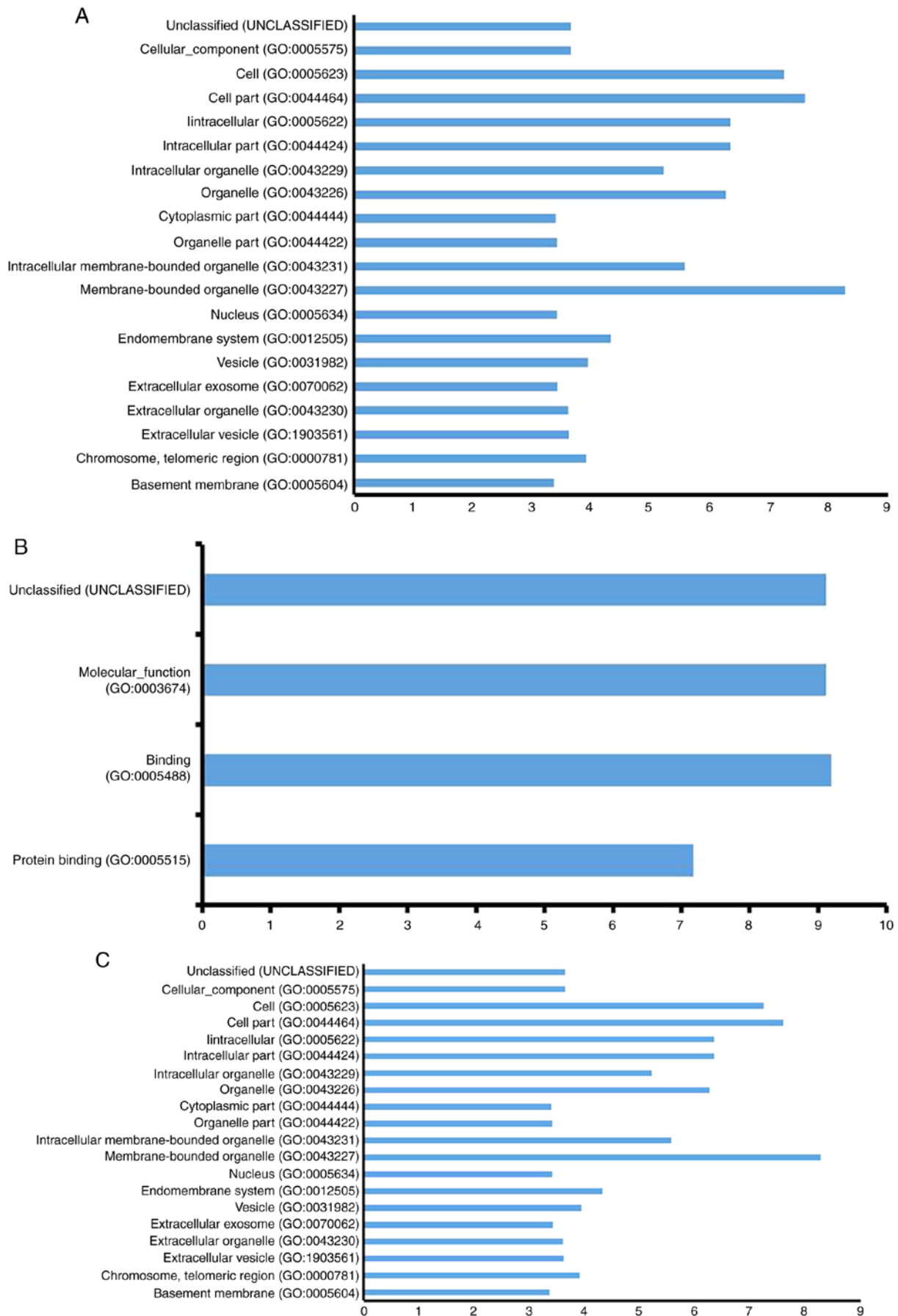
Figure 1. The function of genes collected from Gene Expression Omnibus (GEO) database were determined using geometric means of semantic similarities in biological processes (BPs), molecular functions (MFs), and cellular components (CCs), as measured using the GOSemSim package by considering the Gene Ontology (GO) topological structure in a more precise and unbiased manner. (A) The peak of biological processes (BPs). (B) The peak of molecular functions (MFs). (C) The peak of cellular components (CCs).
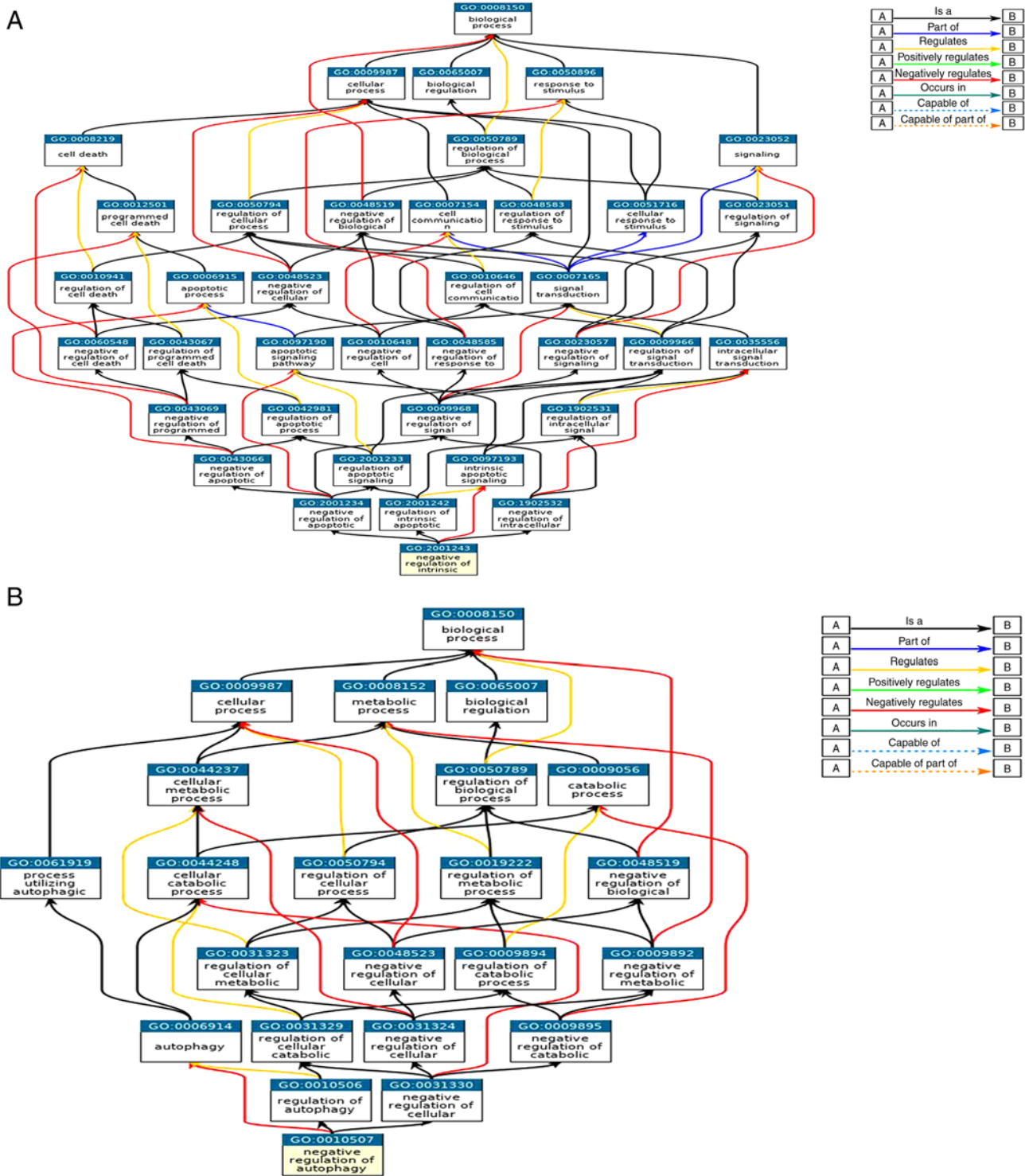
Figure 2. Gene Ontology (GO) items related to the regulation of apoptosis and autophagy. (A) Relationship of GO items in the regulation of apoptosis. (B) Relationship of GO items in the regulation of autophagy.

pathway, the P53 pathway, and others. During analysis, SIRT1 was also found to be related to the AMPK pathway and autophagy pathway, consistent with findings from our previous study (23).

A total of 317 regulator genes, together with lysine-specific demethylase 4A (KDM4A), a regulation protein for the H3K9me3 network containing 220 nodes and 202 edges, were obtained from the STRING online database and Cytoscape software. The PPI network showed detailed protein interactions; however, the PPI enrichment P-value was above 0.05, suggesting that the network did not have significantly more interactions than expected. Nevertheless, the modulation enzyme lysine-specific demethylase 4A (KDM4A) for H3K9me3 could have an interaction with other proteins in the network by HISTIH4C (Histone cluster 1, H4c, a modulation enzyme for histone H4). There were also many protein interactions that could help identify the mechanism of H3K9me3 modulation (Fig. 4).
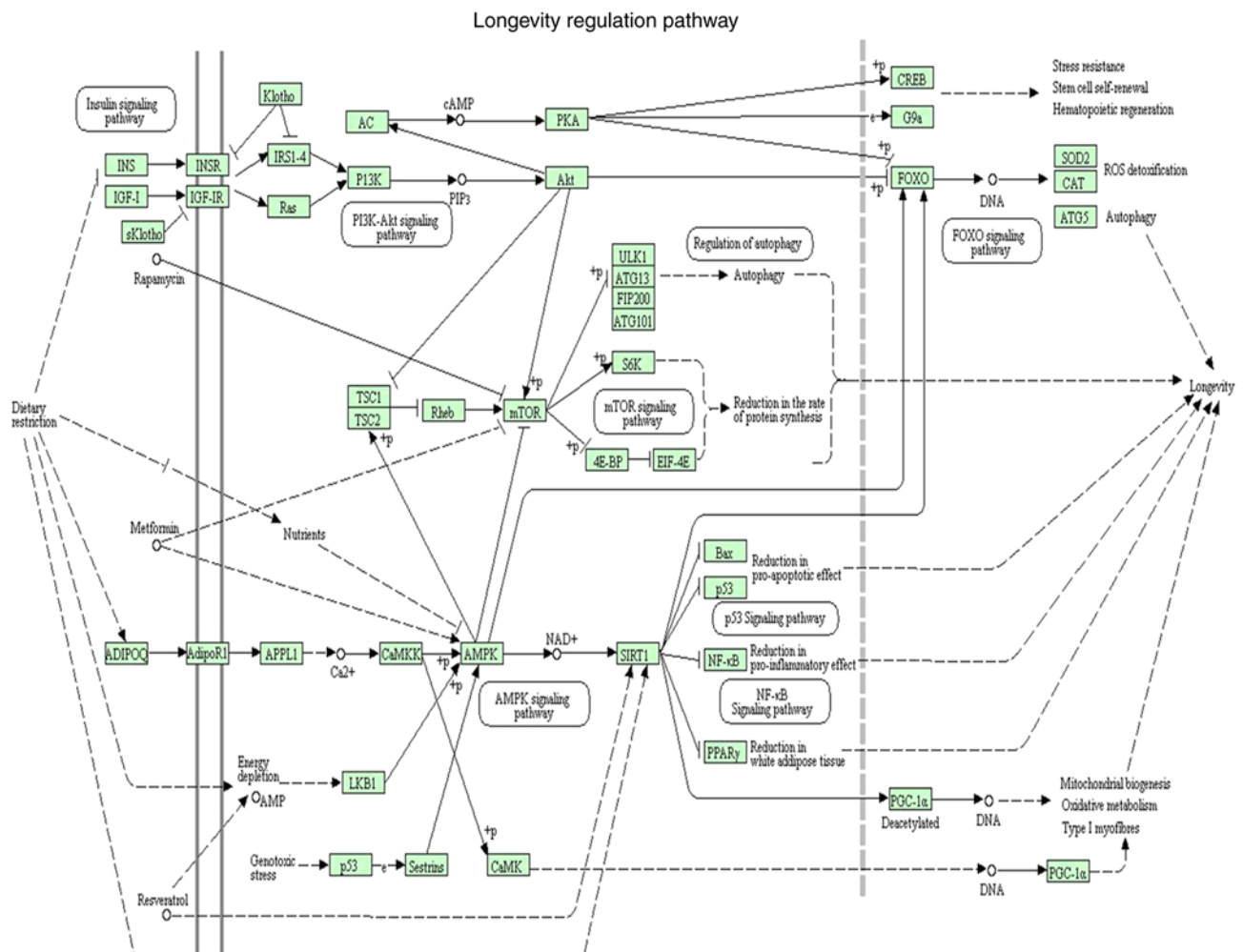
Figure 3. Related pathways were predicted using KEGG Orthology-Based Annotation System (KOBAS) 3.0 online. This pathway was called the longevity pathway. The pathway contained regular pathways related to the survival of leukaemia cells, including the PI3K/AKT pathway, the AMPK pathway, the FOXO pathway, the P53 pathway, mTOR pathway and NF-κB pathway. During analysis, SIRT1 was found to be related to the AMPK pathway or the autophagy pathway, consistent with our previous studies. PI3K, phosphatidylinositol 3-kinase; AKT, serine/threonine kinase; AMPK, protein kinase AMP-activated catalytic subunit α1; FOXO, forkhead box; mTOR, mechanistic target of rapamycin kinase; SIRT1, sirtuin 1.

Distribution of peaks for gene function and the analysis of GO using KEGG network in AML cells treated with chidamide. The ChIP-seq of H3K9me3 was performed in AML cells in which levels of SIRT1 were inhibited by chidamide; this was conducted to identify genes that may be involved in the mechanisms of cell death. The peaks almost clustered in the gene and intergenic areas (nearly 99%); others clustered in areas such as CDS and D10K20K (Fig. 5A).

GO analysis showed that the peak of processes that had significant changes for AML cells treated by chidamide were BPs and MFs. In the BPs, the most significant peaks involved metabolic processes such as the macromolecule metabolic process and cellular macromolecule metabolic processes. In the MFs, the most significant peaks involved metal ion binding or cation binding. KEGG analysis also revealed that the richest peak involved metabolic processes (Fig. 5B).

Network analysis of GO enrichment in AML cells treated by chidamide were in BPs. Units responsive to stress in directed acyclic graph (DAG) had the most significant changes, especially for the DNA repair and cells responsive to stress. In the CCs, the ribosome unit showed the most significant

change; other units also showed significant changes, including cytoplasm and intracellular ribonucleoprotein. In the MFs, phosphotransferase, nucleotide kinase, and adenylate kinase showed the most significant changes.

Our previous research (13) suggested that the level of H3K9me3 could be affected by a low dose of chidamide, thus we aimed to ascertain in the present study whether the network of GO enrichment in AML cells similar to AML patients could be affected by chidamide. This result may be evidence for further research concerning the use of chidamide in AML patients. An interesting result was that the network analysis of GO enrichment in AML cells treated with vhidamide were similar in AML patients. In GO0050789, GO0050794, GO0050896, and GO0080090, all of which are related to BPs, GO enrichment showed significant changes in AML patients compared with control groups; the same also appeared in AML cells treated with chidamide (Fig. 5E), which was different from THP-1 cells without chidamide treatment (Fig. 5C). In the CCs, GO enrichment such as GO005623, GO005622, and GO0044444 showed significant changes in AML patients as well as in the AML cells treated with chidamide. However,
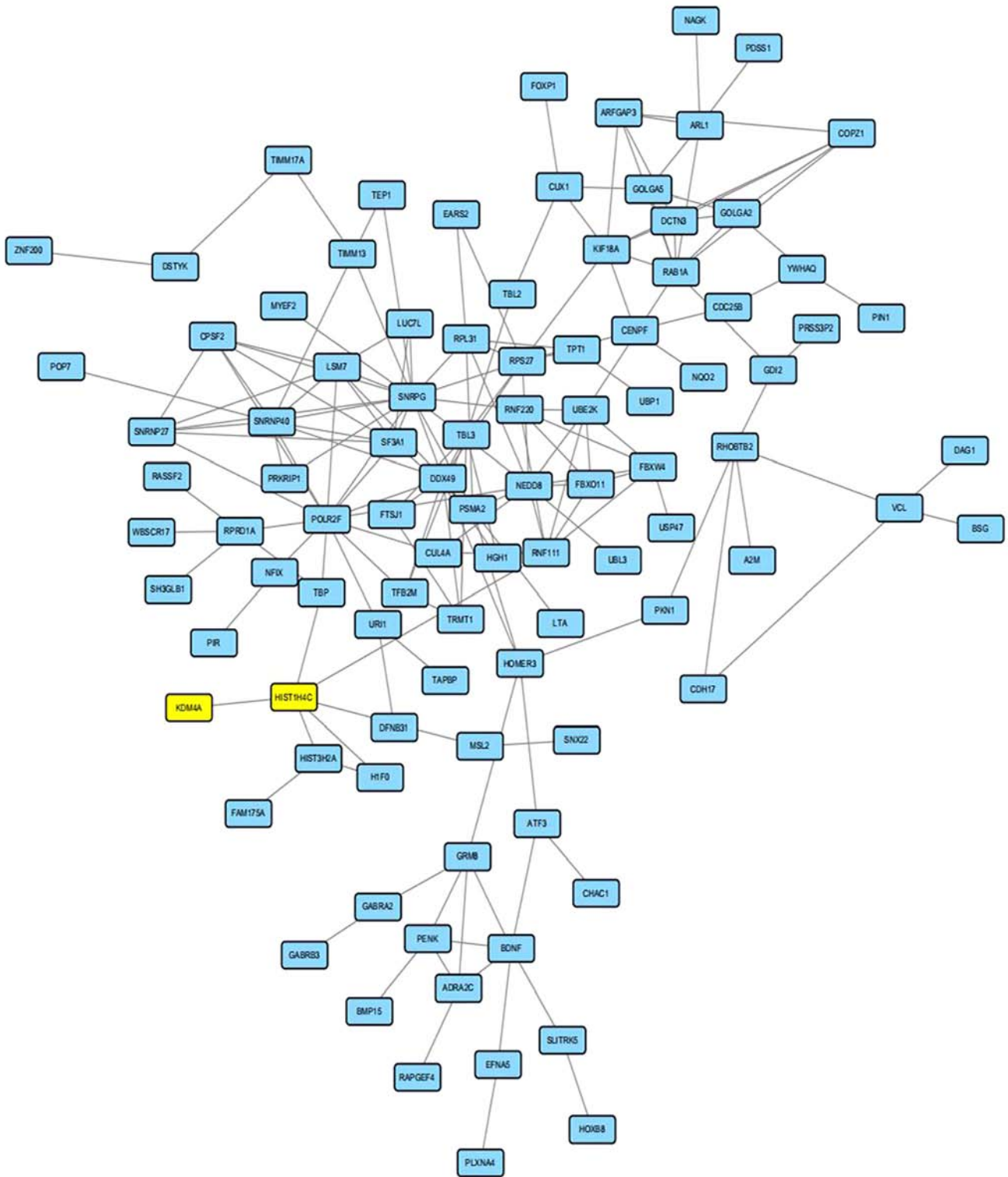
Figure 4. Interaction analysis for regulator genes together with KDM4A demonstrated by software Cytoscape 3.5.1. The protein-protein interaction (PPI) network of regulator genes together with KDM4A showing detailed protein interactions; however, the PPI enrichment P-value was >0.05, suggesting that the network did not have significantly more interactions than expected. KDM4A, lysine-specific demethylase 4A; HISTIH4C, histone cluster 1, H4c.

in MFs, only GO005488 showed significant changes in AML patients and in AML cells treated with chidamide (Fig. 5F), which was different from THP-1 cells without chidamide treatment (Fig. 5D).

Interactions between SIRT1 and H3K9me3 regulatory proteins that showed significant changes in AML patients.

The network of analysis for AML cells treated with chidamide showed several results of GO enrichment that were the same as the results of GO enrichment obtained from the patients in GSE20452. Our previous study showed that SIRT1 (located on chromosome 10) was in the domain of H3K9me3 on chromosome 10 that was downregulated by chidamide, suggesting there may be interaction between SIRT1 and the regulation of
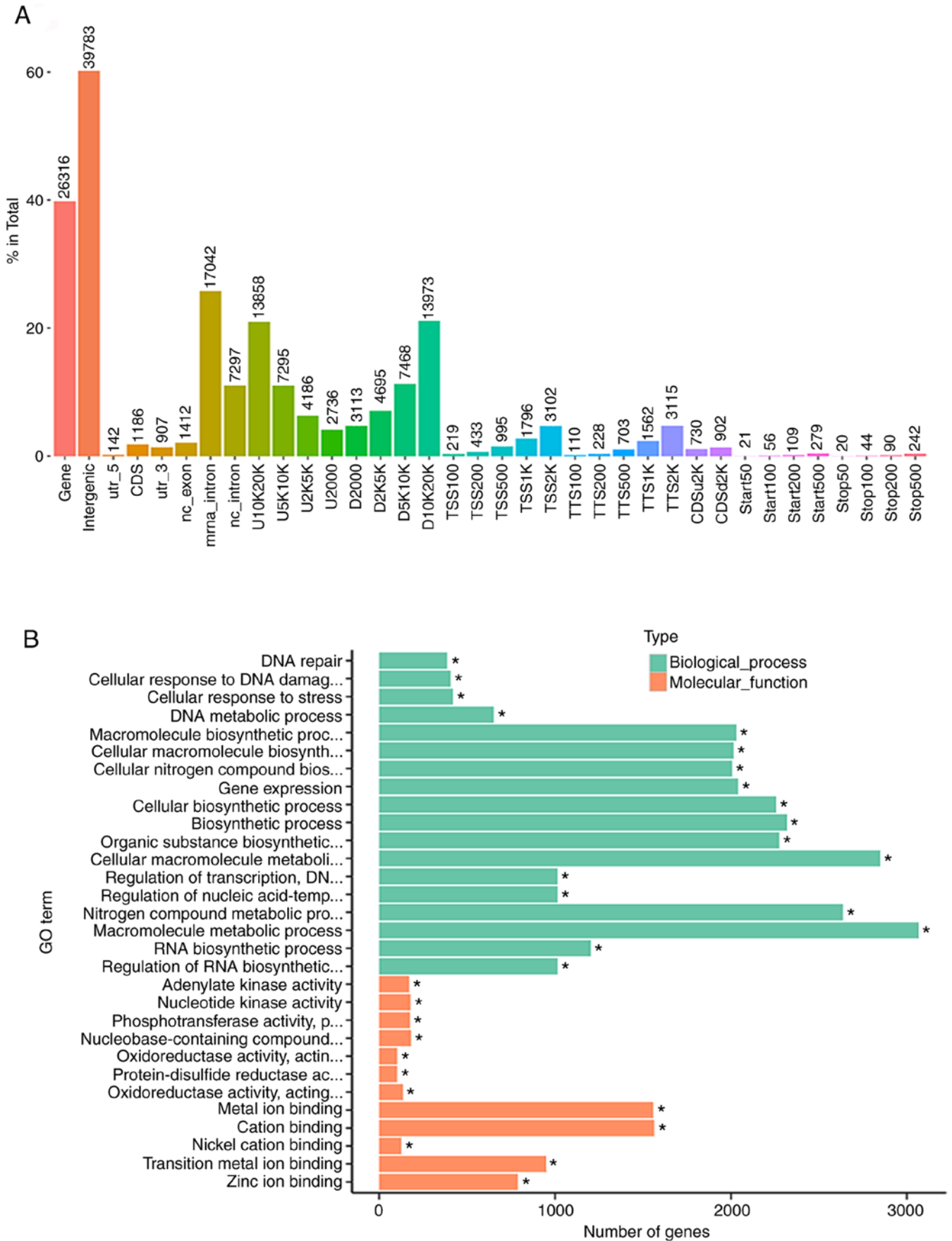
Figure 5. Function of genes collected from acute myeloid leukaemia (AML) cells tested in ChIP-seq were further calculated using the geometric mean of semantic similarities in biological processes (BPs) and molecular functions (MFs), as determined using the GOSemSim package by considering the Gene Ontology (GO) topological structure in a more precise and unbiased manner. (A) The distribution of the peak for gene function. The ratio of the peak almost clustered in the gene and intergenic areas (nearly 99%); others clustered in the areas such as CDS and D10K20K. (B) The peak of biological processes (BPs) and the peak of molecular functions (MFs).
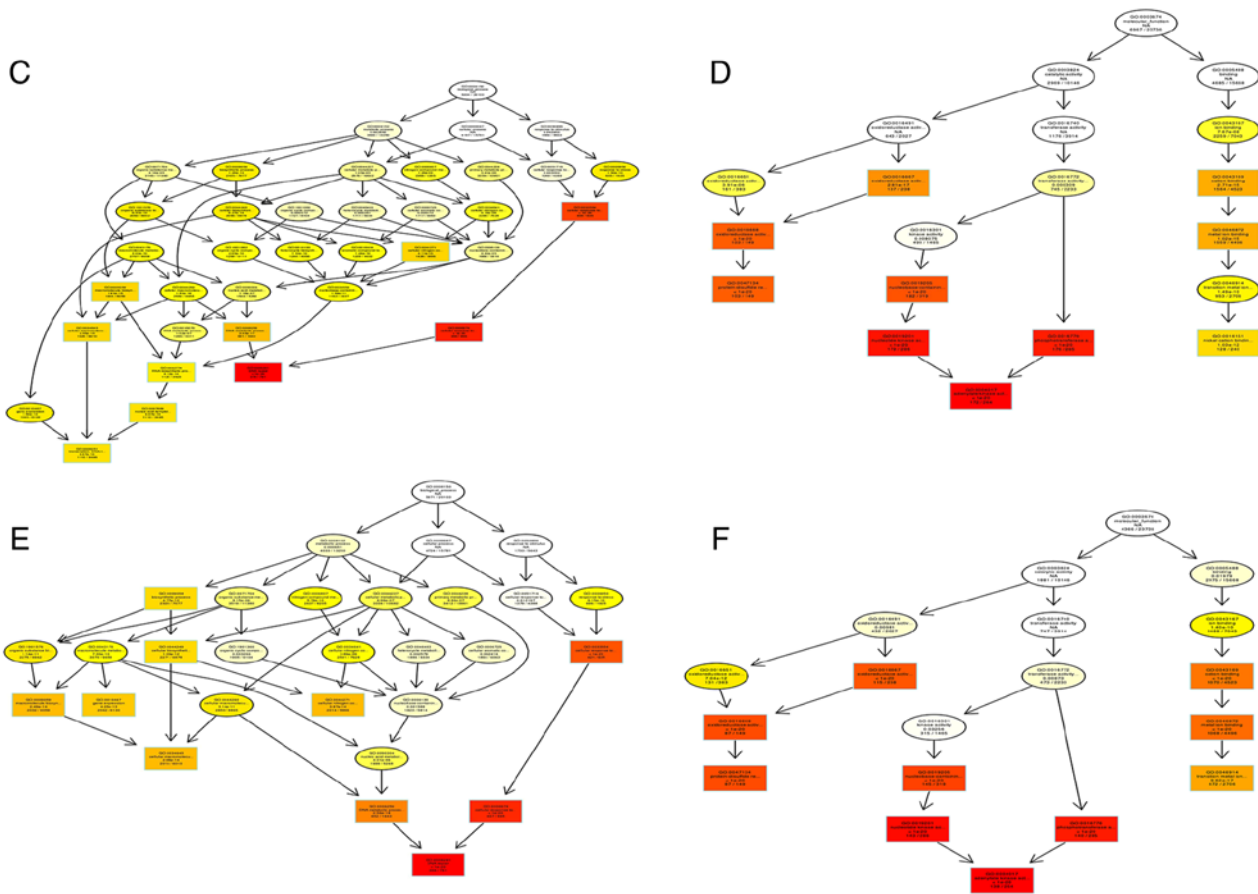
Figure 5. Continued. (C) Relationship of GO items in BPs of THP-1 cells without chidamide treatment. (D) Relationship of GO items in MFs of THP-1 cells without chidamide treatment. (E) Relationship of GO items in the BPs of THP-1 cells treated with chidamide. (F) Relationship of GO items in MFs of THP-1 cells treated with chidamide. In this part, some GO items in BPs or MFs were the same as GO items in AML patients. CDS, CDP-diacylglycerol synthase.

H3K9me3 (17). A total of 317 regulator genes together with SIRT1, containing 219 nodes and 209 edges, were obtained from the STRING online database and Cytoscape software. The PPI network showed detailed protein interactions; however, the PPI enrichment P-value was above 0.05, suggesting that the network did not have significantly more interactions than expected. Nevertheless, SIRT1 had a direct interaction with several proteins in the network compared with KDM4A; however, there were also many other protein interactions that were identified so as to uncover the relationship between SIRT1 and regulatory genes acting on H3K9me3 (Fig. 6A).

Interactions between SIRT1 and KDM4A. Our previous study (13) showed that the location of SIRT1 on chromosome 10 was in the domain of H3K9me3, suggesting there also may exist interactions between SIRT1 and KDM4A. The gene SIRT1, together with KDM4A, contained eight nodes and seven edges, obtained from STRING online database and Cytoscape software. The PPI network showed the detailed protein interactions, and the PPI enrichment P-value was below 0.05, suggesting that the network had significantly more interactions than expected. Nevertheless, there were no direct interactions between SIRT1 and KDM4A. The protein M-phase phosphoprotein 8 (MPHOSPH8) may be the bridge for SIRT1 and KDM4A, and interactions between MPHOSPH8 and KDM4A need to be demonstrated (Fig. 6B).

Discussion

Histone H3 lysine 9 trimethylation (H3K9me3) has a role not only in malignancies but also in normal cellular development. It acts as a repressor of lineage-inappropriate genes and it maintains early cell integrity and genomic stability. In the early 2000s (24), a number of groups provided evidence of its important interactions with evolutionarily conserved amino terminal chromodomain of heterochromatin protein 1 (HP1), a hallmark of heterochromatin, thereby recruiting it to specific chromatin loci. To date, roles for H3K9me3 have been revealed in regulating apoptosis (25,26), autophagy (27), development (28,29), DNA repair (30-33), splicing (34-38), self-renewal (39,40), transcriptional elongation (36), viral latency (41-43), imprinting (44), aging (45), and cell identity (46). In acute myeloid leukaemia (AML), alterations in H3K9 methylation at promoter regions were found to be associated with inactivation of tumour-suppressor genes and blockade of differentiation and deregulated proliferation (47,48). Given the reversible nature of H3K9 trimethylation, this represents an attractive therapeutic target in AML.

Correct identification of the signalling pathways in AML is the foundation of the discovery of therapeutic targets for H3K9me3. In the present study, data from AML patients from the GEO dataset were analysed using GEOR2. Compared with the control group (CD34+ white cells), there were several
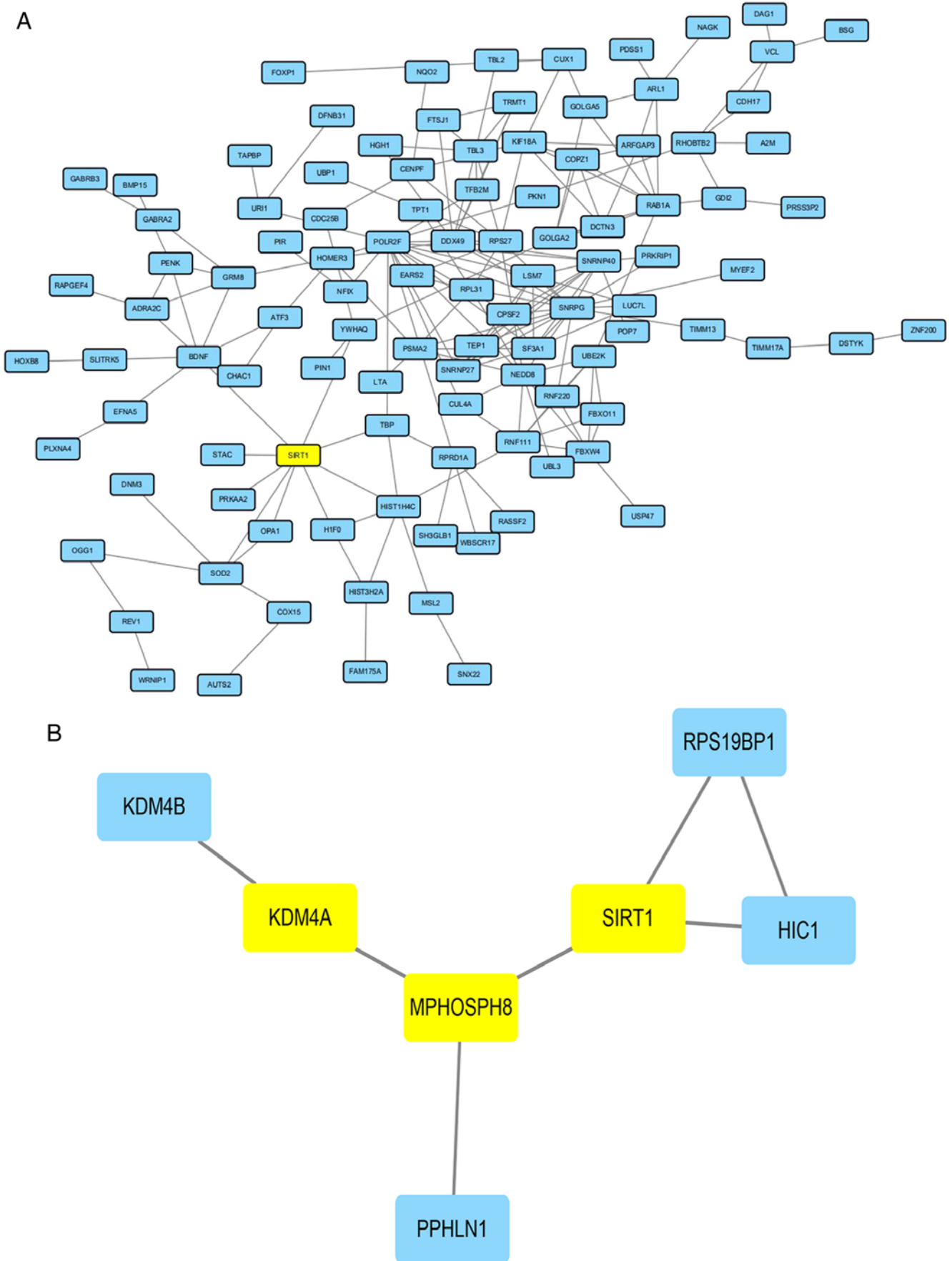
Figure 6. Interaction analysis for regulator genes together with SIRT1 and the interaction analysis for SIRT1 together with KDM4A as demonstrated by software Cytoscape 3.5.1. (A) The PPI network of regulator genes together with SIRT1 showing detailed protein interactions. The PPI enrichment P-value was >0.05, suggesting that the network did not have significantly more interactions than expected. (B) The interaction analysis for SIRT1 together with KDM4A showing that SIRT1 may have an indirect interaction with KDM4A, suggesting that the network had significantly more interactions than expected. KDM4A, lysine-specific demethylase 4A; SIRT1, sirtuin 1.

genes related to changes in H3K9me3 that were significantly differentially upregulated or downregulated. These genes were related to biological processes (BPs), molecular functions (MFs), and cellular components (CCs). Some genes were related to the development of AML and some took part in drug resistance in AML. These changes showed that the change in histone methylation also may be an important factor for the development of AML or drug resistance. In the present study, various changes in THP-1 cells treated with chidamide were the same as that in AML patients. The network analysis of GO enrichment showed that alterations in H3k9me3 may cause changes in cells in regards to apoptosis, autophag. These databases became a potential foundation for our subsequent analysis of chidamide on AML cells *in vitro*. THP-1 is a cell line that is resistant to cytarabine, and chidamide could enhance the cytotoxicity of cytarabine in THP-1 cells by modulating H3K9me3 (13). H3K9me3 was also reported to be related to poor patient prognosis, thus it was thought that H3K9me3 may be related to drug-resistance which is a main factor for poor prognosis (12). There are no studies concerning similar changes in gene expression in other AML cell lines that have been carried out. Changes in the THP-1 cell line in this research may give us a direction for further research in regards to other drug-resistant AML cell lines. The functions of gene expression changes in other drug-resistant AML cells will be carried out in next stage in future research.

The interaction analysis for related proteins of H3K9me3 showed only a few proteins with interactions that had been previously demonstrated in other studies (49-51). The modulation protein lysine-specific demethylase 4A (KDM4A) for H3K9me3 in this analysis was found only with an interaction with HISTIH4C that was a modulation enzyme for another histone (H4). These findings suggest that there are many interactions between KDM4A and other proteins that warrant further investigation. Meanwhile further research concerning the function of KDM4A on AML cells will be carried out in subsequent research.

A recent review highlighted the emerging theme that histone modifications can influence one another, such that one modification recruits or activates chromatin-modifying complexes to generate additional histone modifications (12). Our previous study also showed that the drug chidamide (a histone deacetylation inhibitor (HDACi) developed in China) was the first oral subtype-selective HDACi in the world that could enhance the cytotoxicity of drugs in AML cells (17). One of the potential mechanisms might be related to an effect on H3K9me3. The data suggest that there may be the same changes *in vitro* as those in patients. The ChIP-seq test for THP-1 cells treated with chidamide showed that significant peaks of GO analysis were BPs and MFs, which lack the course of CCs compared with the results in patients. However, the network analysis of GO enrichment *in vitro* found that some changes were related to apoptosis and autophagy. As previously suspected, some forecast changes *in vitro* were the same as the forecast changes in patients, including GO0050789, GO0050794, GO0050896, and GO0080090, related to the BPs or the GO enrichment such as GO005623, GO005622, and GO0044444 in the CCS. These results may be a theoretical basis for further usage of chidamide in AML patients. We believe that these results may suggest target treatments involving H3K9me3.

Changes in H3K9me3 in patients or *in vitro* may cause autophagy. Our previous study regarding the potential mechanisms of action of chidamide in enhancing the cytotoxicity of drugs in AML cells suggested that chidamide inhibits autophagy by inhibiting sirtuin 1 (SIRT1), a histone deacetylation enzyme (17). SIRT1 may also have an interaction with changes in H3k9me3 or the modulation enzyme KDM4A. The pathway of KEGG analysis of H3k9me3 in patients showed several pathways related to the survival of leukaemia cells. These included some related to the survival of leukaemia cells, including the PI3K/AKT pathway, the AMPK pathway, the FOXO pathway, the P53 pathway, and others. SIRT1 had an effect on the FOXO pathway that was downstream of the PI3K/AKT pathway, related to drug resistance. This result may be evidence to support the mechanism of chidamide in reversing drug resistance in AML cells via the *SIRT1* gene. This research is currently being conducted by our research group. In the STRING database analysis, the interaction of SIRT1 with proteins related to a change in H3K9me3 were more evident even more than KDM4A; however, the network did not have significantly more interactions than expected, suggesting that further research needs to be conducted. A compelling result for the interaction of SIRT1 with KDM4A may be a relationship between SIRT1 and KDM4A, although there was not a direct interaction, and there may be involvement of a bridge called the *MPHOSPH8* gene. Research for further verification of the relationship between KDM4A and MPHOSPH8 or SIRT1 must be carried out. This result also suggests a link between histone deacetylation and methylation, as reported in other studies about histone modifications influencing one another (52-55).

In conclusion, bioinformatics analysis of H3k9me3 in patients and in AML cells *in vitro* showed that H3K9me3 may be a target for the treatment for AML; it also suggested that chidamide may be a target drug for AML patients. Finally, our data suggest several directions for the further study of drug resistance in AML.

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Availability of data and materials

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Authors' contributions

AD, WY and WG performed the analysis of the genes for the GEO database peak analysis, as well as analysis of protein interactions.

HH, ZH and XZ performed the ChIP-seq analysis. RY conducted the statistical analysis. WG wrote the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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