Identification of Aberrantly Methylated-differentially Expressed Genes and Potential Agents for Ewing's Sarcoma

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Abstract

Background: DNA methylation is a common epigenetic regulatory way, and it plays a critical role in various human diseases. However, the potential role of how DNA methylation impacts Ewing's sarcoma (ES) is not clear. This study aimed to explore the regulatory role of DNA methylation in ES.

Methods: The microarray data of gene expression and methylation were downloaded from Gene Expression Omnibus (GEO) database, and analyzed via GEO2R. Venn analysis was then applied to identify aberrantly methylated differentially expressed genes (DEGs). Subsequently, Function and pathway enrichment analysis was conducted. Protein-protein interaction (PPI) network was constructed. Hub genes were determined. Besides, a connectivity map (CMap) analysis was performed to screen bioactive compounds for ES treatment.

Results: A total of 135 hypomethylated high expression genes and 523 hypermethylated low expression genes were identified. The hypomethylated high expression genes were enriched in signal transduction and the apoptosis process. Meanwhile, hypermethylated low expression genes were related to DNA replication and transcription regulation. We next determined 10 hub genes through PPI analysis, among them, C3, TF, and TCEB1 might serve as diagnostic and therapeutic targets. Furthermore, CMap analysis revealed 6 chemicals as potential options for ES treatment.

Conclusions: For the first time, we jointly analyzed gene profiling and methylation data about ES. The introduction of DNA methylation characteristics over DEGs is helpful to understand the pathogenesis of ES. The identified hub aberrantly methylated DEGs and chemicals might provide some novel insights on ES treatment.

Background

ES is a malignant tumor that occurs in bone or soft tissue and has a high incidence rate in children and adolescents¹. ES is highly aggressive and easy to relapse after resection². Despite advanced multidisciplinary therapies, the prognosis of ES patients remains poor³. A chromosomal translocation between the *ESR1* gene on chromosome 22 and the *FLI1* gene on chromosome 11 is present in approximately 85% of ES cases⁴. Besides, epigenetic alterations of tumor suppressor genes and protein-coding proto-oncogenes have greatly influenced the development of ES⁵. For all these reasons, research on the epigenetics of ES is highly demanded for improving clinical outcomes.

Epigenetics is related to changes in gene function but does not involve changes in gene sequences⁶. Several molecular factors, including DNA methylation, non-coding RNA, and histones modification, are important in cancer epigenetics, and aberrant DNA methylation has been the subject of intensive research⁷. Aberrant DNA methylation is common in most tumors and greatly influence the process of tumor evolution by influencing the expression of key genes⁸. Although multiple studies have been

performed on aberrant DNA methylation in ES⁹, the comprehensive profile of the aberrantly methylated-differentially expressed genes and their roles in the development of ES remain unclear.

Until now, no research has simultaneously analyzed gene expression and gene methylation data and their link to ES development. In the present study, the gene expression profile (GSE45544) and gene methylation profile (GSE118872) were downloaded from the GEO database, and subsequently, a series of bioinformatics analyses were performed. We identified abnormally methylated-differentially expressed genes and their correlative signaling pathways of ES. The hub genes that might be therapeutic targets were also determined. In the end, we performed a CMap analysis and found several bioactive compounds that might be potential therapeutic agents for ES.

Methods

Microarray data

In this present study, the gene expression profiling datasets (GSE45544) and gene methylation profiling datasets (GSE118872) were downloaded from Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) of the National Center for Biotechnology Information (NCBI). 21 ES and 22 normal samples were included in GSE45544 (platform: GPL6244 Affymetrix Human Gene 1.0 ST Array). Twenty-one ES and 3 normal samples were included in GSE118872 (platform: GPL8490 Illumina Human Methylation 27 Bead Chip).

Data processing

GEO2R, an R-based tool provided in GEO, was used to analyze original submitted data from microarrays. DEGs and DMGs were identified with the criteria of $P < 0.05$ and $|t| > 2$. Subsequently, we obtained hypomethylated-upregulated genes by overlapping hypomethylated and upregulated genes, and by taking the intersection of hypermethylated and downregulated genes, we obtained hypermethylated-downregulated genes.

GO and KEGG enrichment analysis

Functional and pathway enrichment analysis was conducted through the Database for Annotation, Visualization, and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/>)¹⁰. Gene ontology (GO) elucidates the roles of the selected genes in three aspects: molecular function, biological process, and cellular component¹¹. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of selected genes was performed to identify important pathways. P-value < 0.05 was considered significant.

Protein-protein interaction (PPI) network construction and module analysis

To demonstrate the interaction between identified genes, the PPI network was built in the Search Tool for the Retrieval of Interacting Genes (STRING) database with the criteria of an interaction score ≥ 0.4 . For

further analysis, the network was visualized in Cytoscape, an open-source software platform for visualizing complex networks. Molecular Complex Detection (MCODE), an app for cluster analysis in Cytoscape, was used to find important functional modules of selected genes. Degree cut off=2, node score cut off=0.2, k-score=2, and max depth=100 were regarded as significant. CytoHubba, an app in Cytoscape, was used to identify hub genes with the Matthews Correlation Coefficient (MCC) algorithm, and node=5 were set as the criterion.

Identification of small-molecule compounds by CMap analysis

The Connectivity Map (CMap), a gene-expression based drug development database, makes it efficient for researchers to find potential agents that might reverse transcriptional profiles of relevant diseases¹². For CMap analysis, the hub genes were converted into probes and subsequently submitted to the “query” section. P-value <0.01 and enrichment score <0 were set as screening criteria.

Results

Identification of abnormally methylated and differentially expressed genes in ES

To find DEGs or DMGs, we used GEO2R to analyze the corresponding profiling datasets. We then identified 1876 upregulated genes and 6805 downregulated genes from the gene expression microarray (GSE45544). We also obtained 843 hypomethylated genes as well as 1503 hypermethylated genes from gene methylation profiling microarrays (GSE118872). Subsequently, by overlapping 1876 upregulated DEGs and the 843 hypomethylated DMGs, a total of 135 hypomethylated-upregulated genes were gained. Similarly, by taking the intersection of 6805 downregulated DEGs and 1503 hypermethylated DMGs, we obtained 523 hypermethylated-downregulated genes (Fig 1).

GO and KEGG enrichment analysis

To picture the attributes of the genes found above, we played GO and KEGG enrichment analysis through DAVID. For hypomethylated-upregulated genes, we found that the molecular functions (MFs) of those genes were enriched in calcium ion binding, oxidoreductase activity, and serine-type endopeptidase inhibitor activity. As for biological processes (BPs), these genes indicated enrichment in intracellular signal transduction and positive regulation of the apoptotic process. For cell components (CCs), the analysis suggested enrichment mainly focused on extracellular regions. It indicated hypomethylated-upregulated genes might function in the tumor microenvironment of ES. Hypomethylated-upregulated genes might significantly act in some pathways, including metabolic pathways, phagosomes, and peroxisome proliferators-activated receptors (PPAR) signaling pathways (Fig 2).

For hypermethylated-downregulated genes, molecular function enrichment indicated protein binding, DNA binding, and structural constituents of the ribosome. Enriched biological processes included cell division, apoptotic process, and transcription from the RNA polymerase II promoter. Additionally, enriched cell components included the nucleus and nucleoplasm, cytosol and cytoplasm. For hypermethylated-

downregulated genes, the KEGG analysis suggested enrichment of those genes in pyrimidine metabolism, cell cycle, purine metabolism, and ubiquitin-mediated proteolysis (Fig 3).

PPI network construction, module analysis and hub gene selection

To identify the relationships between hypomethylated-upregulated genes, this study conducted PPI network analysis according to the STRING database. The results were visualized in Cytoscape and are shown in Fig 4A. We then performed an MCODE analysis in Cytoscape. Three important modules were obtained and are displayed in Fig 4B-D. The top 5 hub genes were *C3*, *TF*, *PNPLA2*, *VWA1*, and *FUCA2* (Fig 6A). We analyzed hypermethylated-downregulated genes in the same way then the PPI network is displayed in Fig 5A, and three important modules are illustrated in Fig 5B-D. The top 5 hub genes were *CDC20*, *TCEB1*, *CUL2*, *ASB6*, and *KLHL13* (Fig 6B).

CMap analysis

Using the CMap database, we found a total of 6 bioactive compounds that might exert therapeutic effects on Ewing's sarcoma, including iloprost, prednisolone, diphenhydramine, butacaine, benzbromarone, fendiline, as shown in Table 1. Among them, iloprost, benzbromarone, and fendiline were reported with an obvious anti-tumor effect. The others including prednisolone and diphenhydramine were usually combined with other drugs for tumor clinical treatment.

Discussion

Despite progress in the treatment of ES, the survival rate of ES remains poor¹³. Most patients diagnosed with ES have a chromosomal translocation¹⁴. Some researchers have reported that chromosomal translocation can facilitate widespread epigenetic alterations¹⁵. Therefore, it is essential to elucidate the underlying epigenetic changes in ES.

For hypomethylated-upregulated genes, GO enrichment analysis demonstrated that the biological processes were associated with intracellular signal transduction, proteolysis, and positive regulation of apoptotic processes. This is reasonable because previous studies have demonstrated that both altered signal transduction cascades and evasion of apoptosis are hallmarks of cancers, including ES^{16, 17}. PARP proteolysis has been found in the process of apoptosis in Ewing's sarcoma¹⁸. KEGG pathway enrichment analysis showed several significantly enriched pathways including arginine and proline metabolism, the PPAR signaling pathway, and pertussis. The PPAR signaling pathway has been reported to be involved in a variety of cancers, but its specific function is unclear¹⁹. Some researchers believe that PPAR pathway activation could promote the initiation or development of tumors, while others hold the opposite opinion²⁰. Arginine and proline are two important nonessential amino acids, and their synthesis could promote tumor growth²¹. Besides, pertussis toxin can influence the stimulation and inhibition of adenylate cyclase, which plays an important role in Ewing's sarcoma activities²².

Besides, the PPI network illustrated the functional connections between gene expression and gene methylation in ES. Through Cytoscape, the top 5 hub genes were presented: *C3*, *TF*, *PNPLA2*, *VWA1*, and *FUCA2*. The *C3* gene encodes complement C3, which is a central protein associated with most complement activation pathways, and the *C3* gene is a direct target of PPAR receptor α , which could function in certain functional pathways of ES metabolism^{23,24}. The *TF* gene encodes transferrin, an iron-binding transport protein that is usually overexpressed on the surface of tumor cells, including ES cells^{25,26}, some researchers found that transferrin and its receptor were also closely associated with tumor cell growth and proliferation²⁷. The *PNPLA2* gene encodes a protein named adipose triglyceride lipase, which is expressed in human tumors and related to tumor aggressiveness²⁸. The *VWA1* gene encodes von Willebrand factor A-domain-related protein (WARP), the function of which is still unclear²⁹. However, there is translational upregulation of *VWA1* in hepatocellular carcinoma (HCC), and cell proliferation can be inhibited after knockdown of *VWA1*³⁰. The expression of the *FUCA2* can be upregulated by interferon-gamma (IFN- γ), which is closely associated with the initiation and development of tumours³¹. However, little is known about its function in Ewing sarcoma.

For hypermethylated-downregulated genes, GO enrichment analysis demonstrated that the biological processes as cell division, apoptotic process, transcription from the RNA polymerase II promoter, and DNA replication. Molecular function enrichment included protein binding, DNA binding, poly (A) RNA binding and structural constituent of ribosome. KEGG analysis showed that the enriched pathways were pyrimidine metabolism, cell cycle, purine metabolism, and ubiquitin-mediated proteolysis. These results implied the important role of DNA replication and transcription dysregulation in the development of ES, which might be regulated by DNA methylation³².

After the construction of the PPI network, the top 5 hub genes were identified through Cytoscape: *CDC20*, *TCEB1*, *CUL2*, *ASB6*, and *KLHL13*. *CDC20* is important in the regulation of cell cycle, and its expression is upregulated in a variety of malignant tumors^{33,34}. Hypoxia-inducible factor-1 α (HIF-1 α) was found to directly bind to the ES-FLI-1 promoter and could be closely related to the invasiveness of Ewing sarcoma, while *TCEB1* could increase HIF stabilization^{35,36}. Thus, *TCEB1* targeted drug might be helpful for ES treatment. The *CUL2* gene is believed to be a candidate tumor suppressor gene, and the protein it encodes is a member of the cullin family, which is participated in ubiquitination and degradation of certain cellular proteins^{37,38}. The *ASB6* gene encodes a kind of ankyrin repeat protein that contains a C-terminal suppressor of cytokines signaling (SOCS) box motif, and the SOCS functions in ubiquitination and proteasomal degradation of certain proteins³⁹. The Kelch domain-containing protein encoded by *KLHL13* was found to be associated with cytoskeletal functions⁴⁰. The role of *ASB6* and *KLHL13* in the development of ES requires further exploration.

CMap database is commonly used for drug development. In this study, based on CMap analysis, we determined 6 small-molecule compounds that might be potential treatment options for ES. Iloprost has been shown of the highest significance among our results. As a prostaglandin analog, Iloprost has antithrombotic, vasodilatation, and cytoprotection effects⁴¹. In some malignant tumors, increased

platelets were frequently observed, and researchers speculated that those platelets played a critical role in tumor invasion and metastasis⁴². Some preclinical studies demonstrated iloprost could reduce adhesion of tumor cells to platelets, inhibit tumor angiogenesis, and hematogenous spread⁴³, and iloprost significantly inhibited migration and invasion, but not cell growth, in a dose-dependent manner in human ovarian cancer cells by downregulating MMP-2 expression via the IP-mediated PKA pathway. Thus, iloprost might be a potential tumor inhibitor for clinical use. Benzbromarone was also reported with an anti-tumor effect. It might influence the motility of vascular endothelial cells, and subsequently inhibit tumor angiogenesis and cancer metastasis⁴⁴. Fendiline was FDA approved. Recent studies showed fendiline could influence multiple tumor phenotypes, including proliferation, migration, invasion, and independent growth⁴⁵.

In this study, several limitations should be acknowledged. First, we only analyzed two datasets, and a larger sample size is required to verify our results. Also, cell and animal experiments are demanded to further examine the effects of the filtered hub genes and associated pathways on ES. Analysis of clinical parameters and prognosis is also lacking due to the limitations of data. Therefore, further experiments are indispensable to verify the specific function of methylation and hub genes in ES.

Conclusion

In a word, this study identified abnormally methylated-differentially expressed genes and their roles in ES using bioinformatics tools. Besides, we identified 10 hub genes, among them, C3, TF, and TCEB1 might be potential therapeutic targets for ES. The 6 identified bioactive compounds might provide some novel insights for ES treatment.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Shijie Gao, Guowang Li and Hao Yu collected the data and performed the analysis; Shijie Gao, Guowang Li, and Hao Yu wrote the paper; Guangzhi Ning and Wendong Ruan conceived the study. All authors read and approved the final manuscript.

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Tables

Table 1: Results of CMap analysis

Rank	CMap name	Mean	N	Enrichment	P-value
1	iloprost	-0.823	3	-0.964	0.00012
2	prednisolone	-0.371	5	-0.773	0.00106
3	diphenhydramine	-0.444	5	-0.747	0.00192
4	butacaine	-0.35	4	-0.776	0.00515
5	benzbromarone	-0.642	3	-0.834	0.00899
6	fendiline	-0.605	3	-0.831	0.00973

Figures

Figure 1

Venn diagrams showing the aberrantly methylated-differently expressed genes from gene expression microarrays (GSE45544) and gene methylation microarrays (GSE118872) Fig 1A: Hypomethylated and upregulated genes. Fig 1B: Hypermethylated and downregulated genes.

Figure 2

GO and KEGG enrichment analysis of hypomethylated and upregulated genes in ES.

Figure 3

GO and KEGG enrichment analysis of hypermethylated and downregulated genes in ES.

Figure 4

PPI network of hypomethylated-upregulated genes and three modules. Fig 4A: PPI network. Fig 4B: Top 1 module. Fig 4C: Top 2 module. Fig 4D: Top 3 module.

Figure 5

PPI network of hypermethylated-downregulated genes and three modules. Fig 5A: PPI network. Fig 5B: Top 1 module. Fig 5C: Top 2 module. Fig 5D: Top 3 module.

A

B

Figure 6

Hub genes of aberrantly methylated-differently expressed genes in ES. Fig 6A: 5 hub genes of hypomethylated-upregulated genes. Fig 6B: 5 hub genes of hypermethylated-downregulated genes.