

CpG methylation patterns are associated with gene expression variation in osteosarcoma

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Abstract. Osteosarcoma is a common malignant tumor in childhood and adolescence (nearly 5% of all cases of cancer in children), as well as a type of tumor with poor prognosis. However, the pathogenesis and molecular mechanisms of osteosarcoma remains to be elucidated. The aim of the current study was to determine the association between methylation and gene expression changes in osteosarcoma cell line. Microarray data were obtained from the Gene Expression Omnibus database (GSE36004). Genome-wide methylation status was determined in 19 different osteosarcoma cell lines and 6 normal controls. Differentially expressed genes (DEGs) were identified from cancer cells with genefilter package in R and differentially methylated sites were screened with CpGassoc package in R. Integrated gene expression with methylation profiles, genes differentially expressed and methylated, were obtained, and transcriptional regulatory network construction was performed. Functional annotation was performed for genes in the network using the DAVID online tool. Following integrated analysis, a total of 75 methylated sites were demonstrated to be localized at a transcription factor binding region. These sites may be bound by 83 transcription factors which will then alter the expression of 75 downstream DEGs. In the regulatory network, seizure related 6 homolog like 2 (*SEZ6L2*) had the highest degree of upregulation and was demonstrated to be regulated by 12 transcription factors. Furthermore, kin of IRRE like (*KIRREL*), centrosomal protein 72 (*CEP72*) and cyclin-dependent kinase 4 (*CDK4*) were also regulated by more than three transcription factors. Functional annotation revealed that the upregulated genes were primarily involved in the cell cycle pathway. Several differentially methylated sites were associated with upregulation of *SEZ6L2*, *KIRREL*, *CEP72* and *CDK4* and may

have an important role in the pathogenesis of osteosarcomas through promotion of cell proliferation and metastasis.

Introduction

Osteosarcoma, which is most prevalent in childhood and adolescence, is an aggressive, malignant neoplasm that exhibits osteoblastic differentiation and produces malignant osteoid (1). Although the majority of patients are able to have limb surgery, various risk factors, including infection, complications of surgery and local tumor recurrence may induce the need for further surgery (2). In addition, although the 5-year survival rates of patients that received combined treatments, such as chemotherapy and surgery, may be as high as 70%, rates in patients with lung metastasis remain unsatisfactory (20-40%) (3,4). Thus, it is necessary to investigate the pathogenesis and molecular mechanisms of osteosarcoma in further depth to further advance treatment.

To date, various studies have investigated the molecular mechanism of osteosarcoma, including investigation of associated genes and pathways. Of those investigated, the retinoblastoma (*RB*) gene and p53 gene are commonly implicated in the activation of metastatic osteosarcoma (5). Furthermore, it has been demonstrated that the integrity of the *RB* pathway has an important role in tumor behavior, clinical progression and outcome in patients with osteosarcoma (6). Notably, restoration of *RB* also corrected the activity of the p53 pathway in an aggressive osteosarcoma (7,8). A previous study demonstrated that the methylation of heterozygous *RB* and the *RB* promoter have been exhibited in several patients with osteosarcoma (9). Previously, CpG island methylation, which is an epigenetic form of gene regulation that disturbs the function of tumor suppressor genes or oncogenes, has also been demonstrated to contribute to carcinogenesis (10). Skarn *et al* (11) demonstrated that methylation of CpG islands had an important role in the regulation of microRNA expression in osteosarcoma. However, the understanding of the epigenetic alterations implicated in osteosarcoma is currently limited. Therefore, the role of CpG methylation in the pathogenesis and progression of osteosarcoma remains to be defined in full.

A previous study made progress in the investigation of the molecular mechanisms of osteosarcoma dependent on methylation. Kresse *et al* (12) demonstrated the association between the copy number of DNA, mRNA expression and

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DNA methylation in osteosarcoma. Specifically, the present study focused on the effects of CpG island methylation in transcriptional regulation that contributes to the tumorigenesis of osteosarcoma. Based on the DNA methylation and gene expression profiles in osteosarcoma, differentially expressed genes (DEGs) with CpG methylation were identified, and the transcriptional regulatory relationship was analyzed by building a regulatory network. Functional annotation was also performed to investigate the biological role of abnormally expressed genes.

Materials and methods

Data source. Gene expression data of GSE36001 (ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36001) and DNA methylation profiles of GSE36004 (ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36004) were downloaded from the Gene Expression Omnibus (GEO) based on the platform of Illumina human-6 v.2.0 expression BeadChip (Illumina, Inc., San Diego, CA, USA) and Illumina HumanMethylation27 BeadChip (Illumina, Inc.) (12). In each of these datasets, 19 osteosarcoma cell lines were included, while two normal osteoblast cell lines (OB1 and OB2, two primary osteoblast cultures isolated from human calvaria of different donors) were purchased from ScienCell Research Laboratories (California, USA) (12) and four normal bone samples were included as controls. The following osteosarcoma cell lines were included: 143B; HAL; HOS; QIOR/OS9; IOR/OS10; IOR/OS14; IOR/OS15; IOR/OS18; IOR/MOS; IOR/SARG; KPD; MG-63; MHM; MNNG/HOS; OHS; OSA; Saos-2; U-2 OS; and ZK-58. Details of the origin of each cell line or bone sample involved in the datasets can be obtained via the web links provided for each dataset.

Data preprocessing. Raw data of all probes were normalized by the median method (13). Following normalization, the probe name was converted into a gene symbol based on the annotation information. If more than one probe mapped to one gene, an aggregate function in R (14) was performed to calculate the mean expression value for this gene. Probes with missing values were imputed with the nearest neighbor averaging method (15) of imputation (impute) package (version 1.0; <https://bioconductor.org/packages/release/bioc/html/impute.html>) in R (16). The DNA methylation data obtained from GEO was preprocessed with BeadStudio software (version 3.1) from Illumina, Inc. where the methylated locus for each sample with a missing value was removed.

Identification of DEGs in osteosarcoma cells. To identify DEGs between osteosarcoma cells and normal controls, one way analysis of variance (ANOVA) in the genefilter package of R was performed (15). P-values were generated using Benjamini-Hochberg (BH) multiple testing correction method and $P < 0.05$ was considered to indicate a statistically significant difference. The ratio of mean expression of normal and osteosarcoma cell groups was used to determine whether genes were up or downregulated.

Identification of disease-associated methylated regions (DMR). To identify disease-associated CpG methylated sites

in osteosarcoma cells, compared with normal control for disease-association analysis, CpG loci with beta values that were not significantly different between case and control were eliminated. Similarly, all CpG loci on the X, Y and mitochondrial chromosomes were removed. Subsequently, the CpGassoc package of R (17) was used for disease-association analysis, which is designed to investigate the association between methylation at CpG loci across the genome and a phenotype of interest. CpGassoc algorithm of R package was used to determine the association between CpG loci and osteosarcoma. In addition, CpGassoc can also be used to create quantile-quantile plots, manhattan plots and scatterplots for individual CpG sites. BH multiple testing correction was used to estimate the false discovery rate (FDR) in disease-associated analysis. $FDR \leq 0.05$ was chosen as the threshold.

Integration analysis of gene expression and methylation profiles. To investigate the association between methylation and gene expression in osteosarcoma cell lines, methylation data was measured on ± 2 kb genomic regions around the transcriptional start sites (TSS) of each gene. The obtained genes were differentially methylated. Integration analysis was performed to identify genes where there was an overlap between differential expression between control and osteosarcoma, and the presence of methylation. In order to investigate the influence of methylation on gene expression, the transcription factor binding sites were searched within the UCSC database (18). Subsequently, methylated DEGs in transcription factor binding regions in osteosarcoma cells were screened.

Construction of transcriptional regulatory networks. Based on the transcription factor and target gene information provided by the UCSC database, transcriptional regulatory networks were constructed and further visualized by Cytoscape software (version 2.8.0; www.cytoscape.org) (19). In the network, the node degree was calculated by igraph package in R (20).

Functional annotation of target genes of transcription factors. To understand the biological roles of target genes of the transcription factors blocked by DNA methylation, gene ontology (GO) function and pathway enrichment analysis was performed by DAVID (database for annotation, visualization, and integrated discovery) online tool (21). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Identification of DEGs in osteosarcoma cells. Following preprocessing of methylation profile data, a total of 20,006 methylation sites were identified from the 25 samples. Similarly, a total of 24,214 genes were identified from 25 samples following preprocessing of expression profile data. By applying ANOVA, a total of 6,419 DEGs were identified in osteosarcoma cells compared with normal control, including 3,236 upregulated and 3,183 downregulated genes.

Identification of DMRs. To identify differentially methylated genes, the present study performed three types of analyses. Initially preprocessing of raw methylation data was performed and 20,006 methylated sites were identified in the 25 samples

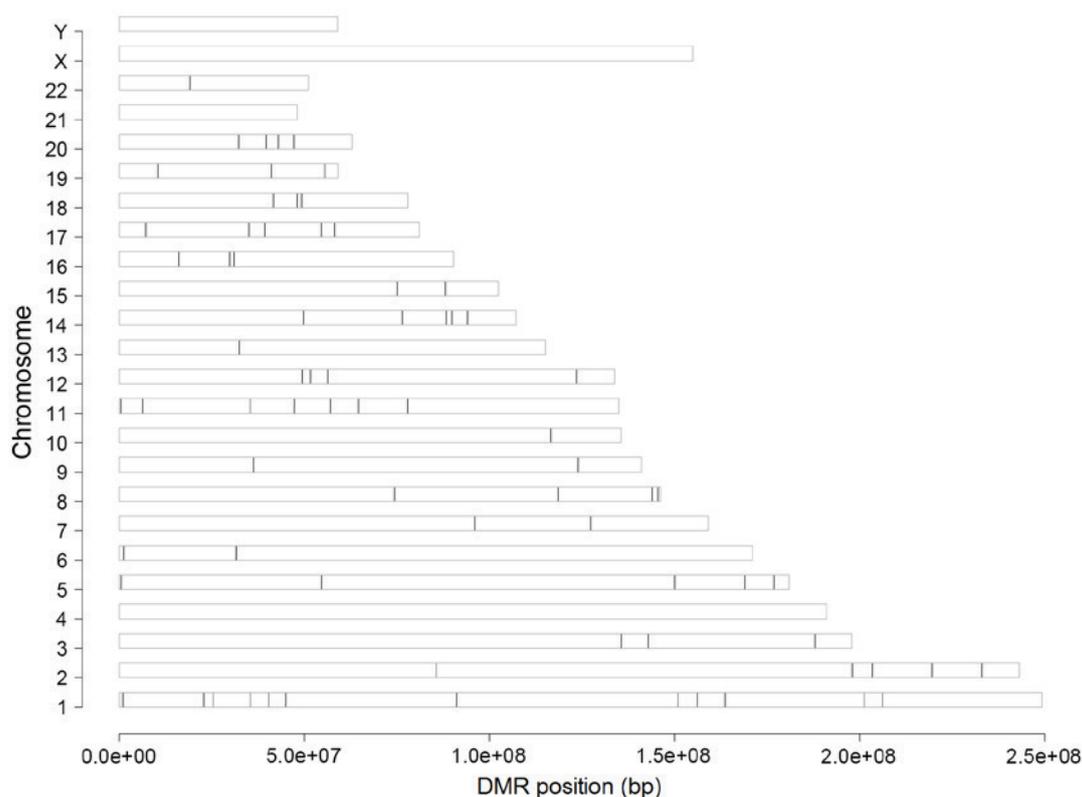


Figure 1. Distribution of DMRs on chromosomes. The y axis indicates chromosome location and the x axis indicates the position of DMRs. All DMRs located on the X, Y and mitochondrial chromosomes were removed. DMRs, disease-associated methylated regions.

included in the dataset. Subsequently, methylated sites that were located on the X, Y and mitochondrial chromosomes and imputation analysis was performed; as a result, 5,921 sites were eliminated from further analysis. Finally, following disease-associated analysis, 13,750 differentially methylated genes located in different chromosome regions, with an $FDR < 0.05$, were identified in osteosarcoma cells (Fig. 1).

Integration analysis of DEGs and differentially methylated genes. Integration analysis indicated that 3,625 genes were differentially expressed in osteosarcoma and control, and also methylated around their TSS. Based on the UCSC database for transcription factors, a total of 75 methylated sites were located in the transcription factor binding regions, which may affect 83 transcription factors and 75 downstream target genes.

Construction of transcription regulatory network. Based on different types of regulation, a transcription regulatory network was constructed, which included 83 transcription factors and 75 downstream target genes (Fig. 2). In the network, there were 158 nodes and 129 edges. Of the genes in the network, the overexpressed gene seizure related 6 homolog like 2 (*SEZ6L2*) had the highest degree. It was observed that *SEZ6L2* was regulated by 12 transcription factors, including signal transducer and activator of transcription 3 (*STAT3*) and early growth response 1-3 (*EGR1-3*). Additionally, the transcription factor myocyte enhancer factor 2 (*MEF2*), which had a higher degree compared with other transcription factors, regulated seven downstream targets, including the upregulated replication factor C (activator 1) 4, cyclin-dependent kinase 4 (*CDK4*)

and chromodomain helicase DNA binding protein 6 genes, and downregulated the chromosome 14 open reading frame 102 gene. Similarly, the upregulated kin of IRRE like gene (*KIRREL*) was regulated by seven different transcription factors. Transcription factor paired box 5 (*PAX5*) was indicated to be involved in the regulation of three up and two downregulated genes in the network. Upregulated genes, centrosomal protein 72 (*CEP72*), block of proliferation 1 (*BOPI*) and TruB pseudouridine synthase family member 1, were regulated by a different set of three transcription factors: *STAT3*, *MEF2* and adaptor-related protein complex 1 (*API*).

Functional annotation of target genes of transcription factors. GO functional enrichment analysis was performed for 46 upregulated and 29 downregulated genes. The top five enriched categories are listed in Tables I and II. The upregulated genes were predominantly enriched in the GO terms of cell cycle, non-coding RNA metabolic process and the maturation of large subunit ribosomal RNA (rRNA) from tricistronic rRNA transcript. In addition, downregulated genes were predominantly involved in the inflammatory response, regulation of the humoral immune response and response to wounding.

Discussion

Osteosarcoma is the most common histological form of primary bone cancer. To analyze the effects of genome-wide changes in gene expression and DNA methylation in osteosarcoma cell lines, the present study identified 75 significantly

Table I. GO function and pathway enrichment analysis of upregulated genes in the transcriptional regulatory network.

Term		Count	P-value
ID	Name		
REACTOME_PATHWAY			
REACT_152	Cell cycle, mitotic	4	0.028263722
GOTERM_BP_FAT			
GO:0034660	ncRNA metabolic process Maturation of LSU-rRNA	5	0.002014149
GO:0000463	from tricistronic rRNA transcript (small subunit-rRNA, 5.8S rRNA and LSU-rRNA)	2	0.007080142
GO:0000470	Maturation of LSU-rRNA	2	0.007080142
GO:0006396	RNA processing	6	0.008649471
GO:0034470	ncRNA processing	4	0.009603024
GOTERM_CC_FAT			
GO:0070013	Intracellular organelle lumen	12	0.002136088
GO:0043233	Organelle lumen	12	0.002571635
GO:0031974	Membrane-enclosed lumen	12	0.00301231
GO:0005654	Nucleoplasm	8	0.004426136
GO:0031981	Nuclear lumen	10	0.006006103
GOTERM_MF_FAT			
GO:0005524	ATP binding	9	0.019812363
GO:0032559	Adenyl ribonucleotide binding	9	0.021338813
GO:0008026	ATP-dependent helicase activity	3	0.022750064
GO:0070035	Purine nucleotide triphosphate-dependent helicase activity	3	0.022750064
GO:0042623	ATPase activity, coupled	4	0.026532158

REACT, reactome; GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function; ncRNA, non-coding RNA; LSU-rRNA, large subunit ribosomal RNA.

Table II. Functional annotation of downregulated genes in the transcriptional regulatory network.

Term		Count	P-value
ID	Name		
GOTERM_BP_FAT			
GO:0006954	Inflammatory response	4	0.01325742
GO:0002920	Regulation of humoral immune response	2	0.018477257
GO:0009611	Response to wounding	4	0.047076136
GOTERM_MF_FAT			
GO:0005496	Steroid binding	3	0.004729209

GO, gene ontology; BP, biological process; MF, molecular function.

in human osteosarcoma cells (33). Furthermore, it has been demonstrated that GATA2 is required for proliferation in various cancer cell types (34). Thus, differential expression of *KIRREL* may be regulated by methylation and transcription factors that promote tumor development. In addition, *CEP72*, *CDK4* and *BOPI* were differentially methylated and regulated by transcription factors, including STAT3, MEF2 and AP1. *CEP72* encodes a protein that is a member of the leucine-rich-repeat superfamily. *CEP72* has been identified

as possessing a high incidence of genomic copy number changes in the 5p15.33 region in patients with non-small cell lung cancer (35). As osteosarcoma has a high tendency for metastatic spread and predominantly arises in the lungs, *CEP72* may have a key role in cancer metastasis. In addition, it was previously demonstrated that *CEP72* was regulated by AP1, which is involved in the ERK signaling pathway (36). It is well established that CDKs are essential drivers of cell cycle progression and are commonly dysregulated during

tumorigenesis (37). CDK4 and other CDK inhibitors have been identified as a class of promising anticancer agents in cancer treatment (38). Furthermore, regulated transcription factors STAT3 and API have been demonstrated to be involved in cancer development and progression via promotion of the cell cycle (39,40). The functional annotation performed in the present study was consistent with a previous study about the enriched pathway of *CEP72* and *CDK4* (41).

In conclusion, methylation of *SEZ6L2*, *KIRREL*, *CEP72* and *CDK4* may have an important role in the pathogenesis of osteosarcomas through promotion of cell proliferation and metastasis. However, further study into the results is required and may provide further insight into the molecular mechanism of osteosarcoma. In addition, further experiments, including western blot analysis and reverse transcription-polymerase chain reaction will be performed in the future to validate the changes in gene expression.

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