G&I Genomics & Informatics

eISSN 2234-0742
Genomics Inform 2014;12(4):247-253
http://dx.doi.org/10.5808/GI.2014.12.4.247

ORIGINAL ARTICLE

Analysis of Gene Expression in Cyclooxygenase-2-Overexpressed Human Osteosarcoma Cell Lines

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Osteosarcoma is the most common primary bone tumor, generally affecting young people. While the etiology of osteosarcoma has been largely unknown, recent studies have suggested that cyclooxygenase-2 (COX-2) plays a critical role in the proliferation, migration, and invasion of osteosarcoma cells. To understand the mechanism of action of COX-2 in the pathogenesis of osteosarcoma, we compared gene expression patterns between three stable COX-2-overexpressing cell lines and three control cell lines derived from U2OS human osteosarcoma cells. The data showed that 56 genes were upregulated, whereas 20 genes were downregulated, in COX-2-overexpressed cell lines, with an average fold-change > 1.5. Among the upregulated genes, *COL1A1*, *COL5A2*, *FBN1*, *HOXD10*, *RUNX2*, and *TRAPPC2* are involved in bone and skeletal system development, while *DDR2, RAC2, RUNX2,* and *TSPAN31* are involved in the positive regulation of cell proliferation. Among the downregulated genes, *HIST1H1D, HIST1H2AI, HIST1H3H*, and *HIST1H4C* are involved in nucleosome assembly and DNA packaging. These results may provide useful information to elucidate the molecular mechanism of the COX-2-mediated malignant phenotype in osteosarcoma.

Keywords: cell proliferation, cyclooxygenase 2, invasion, osteosarcoma, overexpression, migration

Introduction

Osteosarcoma is the most common primary bone tumor, representing over 56% of all bone tumors. It generally affects young people with the age of 15 to 19 and usually occurs in long bones near the metaphyseal growth plates. It has a high tendency to metastasize, and about 10%–20% of patients have macroscopic evidence of metastasis at the time of diagnosis, while 80%–90% of patients are assumed to have micrometastasis. Although it has been postulated that rapid bone growth, exposure to radiation, and genetic predispositions, such as *RB* or *p53* mutations, are thought to be risk factors for developing osteosarcoma, the etiology has not been fully understood [1-3].

Prostaglandin endoperoxide synthase 2 (PTGS2), also called as cyclooxygenase-2 (COX-2), catalyzes the convertsion of arachidonic acid to prostaglandin H2, from which various prostanoids, including prostaglandin E_2, are produced [4].

Accumulating evidence indicates that COX-2 is involved in osteosarcoma development and progression. Several studies have reported that high levels of COX-2 expression is associated with advanced clinical stage and metastasis [5, 6], as well as with lower overall survival rates and disease-free survival rates [7-9]. In addition, COX-2 inhibition by using RNAi or antisense oligonucleotide inhibits cell proliferation and invasion in human osteosarcoma cells [10, 11]. Also, selective COX-2 inhibitors reduce not only osteosarcoma cell proliferation and invasion *in vitro* but also tumor growth and metastasis in vivo [12, 13]. Moreover, we have previously reported that COX-2 overexpression promotes cell

Received October 20, 2014; Revised November 9, 2014; Accepted November 10, 2014

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proliferation, migration, and invasion in U2OS human osteosarcoma cells [14]. These studies strongly suggest that COX-2 might be a causal factor for the development and progression of osteosarcoma. However, the exact mechanisms of action of COX-2 in osteosarcoma are largely unknown.

In an attempt to figure out the mechanism of action of COX-2 in osteosarcoma, we analyzed the gene expression profiles in three COX-2-overexpressed U2OS stable cell lines and three control stable cell lines.

Methods

Establishment and maintenance of stable cell lines

Human COX-2 cDNA was subcloned into the pcDNA3 vector containing neo^r. U2OS cells were transfected with COX-2 or pcDNA3 DNA using Lipofectamine2000 (Life Technologies, Grand Island, NY, USA). Transfectants were selected in the presence of geneticin, and individual clones were maintained in Dulbecco's modified Eagle's medium, containing fetal bovine serum (10%), penicillin (100 units/mL), streptomycin (100 units/mL), and geneticin (700 μg/mL), as reported previously [14].

RNA isolation

Total RNA was extracted from cells with Trizol (Life Technologies), purified with the addition of chloroform, and precipitated with the addition of isopropanol. The RNA concentration was determined by a spectrophotometer, and the quality of RNA was evaluated by the OD 260/280 ratio and gel electrophoresis.

Hybridization to expression arrays

The following procedures were carried out by Macrogen Co. (Seoul, Korea). First, total RNA was amplified and purified using the Ambion Illumina RNA amplification kit to yield biotinylated cRNA (Ambion, Austin, TX, USA). Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo(dT) primer. Second-strand cDNA was synthesized, in vitro-transcribed, and labeled with biotin-NTP. After purification, 750 ng of labeled cRNA was hybridized to the humanHT-12 expression v.4 bead array (Illumina, San Diego, CA, USA) for 16-18 h at 58°C. The array signal was detected using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK). Arrays were scanned with an Illumina bead array reader/confocal scanner. Array data were filtered by a detection p-value $<$ 0.05 (similar to signal to noise). Selected gene signal values were log-transformed and normalized by the quantile method.

Statistical analysis

Basic statistical analyses were performed using Microsoft Excel. Hierarchical cluster analysis was conducted with normalized log2-gene expression values using Cluster 3.0, and the results were visualized using Java Treeview [15, 16]. An unrooted tree was drawn with R package. Biological function analysis was performed with official gene names using DAVID (http://david.abcc.ncifcrf.gov/).

Results

Stable cell lines

We have previously established stable cell lines overexpressing human COX-2 in U2OS human osteosarcoma cells. To avoid clonal variations, we established three stable COX-2-overexpressing cell lines (U2OS-COX-2 #1, #2, and #3) and three control cell lines (U2OS-pcDNA3 #1, #2, and #3). High levels of COX-2 expression were observed by western blot analysis in U2OS-COX-2 cells, whereas the expression was barely detectable in U2OS-pcDNA3 cells. When we cultured the stable cell lines, we observed that cell proliferation, migration, and invasion rates were increased significantly in U2OS-COX-2 cells as compared with U2OS-pcDNA3 cells [14] (Table 1).

Gene expression

We first performed unsupervised cluster analysis comparing COX-2-overexpressing cell lines and control cell lines. Among 24,371 probe sets, we selected and analyzed 567

Table 1. Summary of stable cell line characteristics

Cell line	Expression level of COX-2	Proliferation rate	Migration rate (trans-well)	Invasion rate (Matrigel)
U2OS-COX-2 #1	+++	+++	+++	+++
U2OS-COX-2 #2	+++	+++	+++	+++
U2OS-COX-2 #3	+++	+++	+++	+++
U2OS-pcDNA3 #1	±	+	+	+
U2OS-pcDNA3 #2	±	+	+	+
U2OS-pcDNA3 #3	±	+	+	+

COX-2, cyclooxygenase-2.

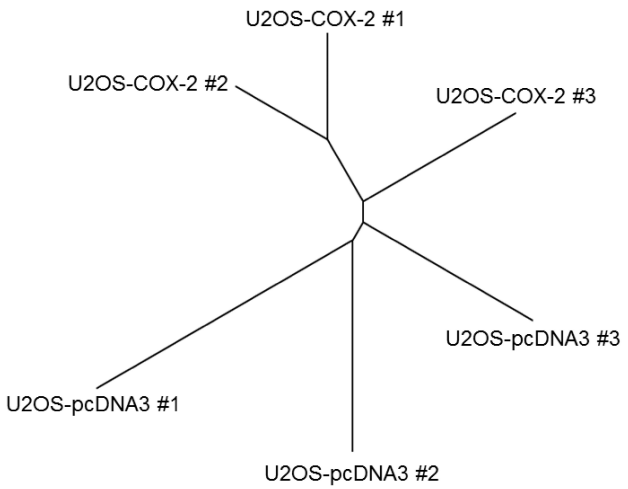

Fig. 1. Segregation between the group of U2OS-COX-2 and the group of U2OS-pcDNA3. Unsupervised cluster analysis was done between six stable cell lines using 567 probe sets with a standard deviation > 0.5. Normalized $\log_2$-gene expression values were used for hierarchical clustering, and an unrooted tree was drawn by R package. COX-2, cyclooxygenase-2.

probe sets with a standard deviation > 0.5. The group of U2OS-COX-2 (U2OS-COX-2 #1, #2, and #3) and the group of U2OS-pcDNA3 (U2OS-pcDNA3 #1, #2, and #3) were well segregated in the unsupervised cluster analysis, confirming that our experiment was validated (Fig. 1).

To identify significantly differentially expressed genes (DEGs) in COX-2-overexpressing osteosarcoma cells, we first performed the Wilcoxon rank sum test between the group of U2OS-COX-2 and the group of U2OS-pcDNA3. The 2,144 probe sets passed the criteria. Then, to identify DEGs, we selected 76 probe sets with an average fold-change > 1.5.

Among the DEGs, 56 genes were upregulated while 20 genes were downregulated in the group of U2OS-COX-2 as compared to the group of U2OS-pcDNA3 (Tables 2 and 3). The heat map of DEGs is provided in Fig. 2. The gene encoding COX-2, PTGS2, was upregulated by 10.41-fold in the COX-2-overexpressing cell lines as compared to the control cell lines, confirming again that our experiment is

Table 2. Upregulated DEGs in COX-2-overexpressed cell lines

Symbol	Description	Fold change	Accession No.
PTGS2	Prostaglandin-endoperoxide synthase 2	10.41	NM_000963
PAGE2B	P antigen family, member 2B	4.61	NM_001015038
COL1A1	Collagen, type I, alpha 1	3.21	NM_000088
FAP	Fibroblast activation protein, alpha	2.76	NM_004460
COL5A2	Collagen, type V, alpha 2	2.35	NM_000393
LINC00312	Long intergenic non-protein coding RNA 312	2.34	NR_024065
RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP-binding protein Rac2)	2.33	NM_002872
HOXD10	Homeobox D10	2.32	NM_002148
RCN3	Reticulocalbin 3, EF-hand calcium-binding domain	2.07	NM_020650
FBN1	Fibrillin 1	2.02	NM_000138
CREB3L1	cAMP responsive element-binding protein 3-like 1	2.02	NM_052854
C1orf85	Chromosome 1 open reading frame 85	2.01	NM_144580
TSPAN31	Tetraspanin 31	2.01	NM_005981
DPYSL5	Dihydropyrimidinase-like 5	1.96	NM_020134
PSMB9	Proteasome (prosome, macropain) subunit, beta type, 9, transcript variant 1	1.89	NM_002800
PSMB9	Proteasome (prosome, macropain) subunit, beta type, 9, transcript variant 2	1.89	NM_148954
GPAM	Glycerol-3-phosphate acyltransferase, mitochondrial	1.89	NM_020918
NLRP1	NLR family, pyrin domain-containing 1, transcript variant 1	1.83	NM_033004
LTBP1	Latent transforming growth factor beta-binding protein 1, transcript variant 1	1.82	NM_206943
UNC13A	Unc-13 homolog A (*Caenorhabditis elegans*)	1.79	NM_001080421
VAT1L	Vesicle amine transport 1-like	1.78	NM_020927
MEIS1	Meis homeobox 1	1.77	NM_002398
DDR2	Discoidin domain receptor family, member 2, transcript variant 1	1.74	NM_001014796
DYSF	Dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	1.74	NM_003494
ACBD7	Acyl-coenzyme A-binding domain-containing 7	1.69	NM_001039844
FMN1	Formin 1	1.68	NM_001103184
SCN3A	Sodium channel, voltage-gated, type III, alpha subunit, transcript variant 1	1.68	NM_006922
NLRP11	NLR family, pyrin domain-containing 11	1.68	NM_145007
MBOAT2	Membrane bound O-acyltransferase domain-containing 2	1.68	NM_138799

DEG, differentially expressed gene; COX-2, cyclooxygenase-2.

Table 2. Continued

Symbol	Description	Fold change	Accession No.
MIR196A1	MicroRNA 196a-1	1.67	NR_029582
ASTN1	Astrotactin 1, transcript variant 1	1.65	NM_004319
TSHZ2	Teashirt family zinc finger 2	1.64	NM_173485
MFAP2	Microfibrillar-associated protein 2, transcript variant 2	1.64	NM_002403
SH3PXD2A	SH3 and PX domains 2A	1.62	NM_014631
SNORD116-22	Small nucleolar RNA, C/D box 116-22	1.60	NR_003336
TSHZ2	Teashirt zinc finger homeobox 2	1.59	NM_001193421
EHD3	EH domain-containing 3	1.59	NM_014600
LBH	Limb bud and heart development homolog (mouse)	1.59	NM_030915
PTPN20B	Protein tyrosine phosphatase, non-receptor type 20B	1.59	NM_001042357
C10orf11	Chromosome 10 open reading frame 11	1.59	NM_032024
SNORD116-2	Small nucleolar RNA, C/D box 116-2 on chromosome 15	1.59	NR_003317
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1, transcript variant 1	1.59	NM_004105
NAG	Neuroblastoma-amplified protein	1.58	NM_015909
HIST4H4	Histone cluster 4, H4	1.58	NM_175054
KIF5A	Kinesin family member 5A	1.57	NM_004984
TMEPAI	Transmembrane, prostate androgen-induced RNA, transcript variant 1	1.56	NM_020182
UACA	Vveal autoantigen with coiled-coil domains and ankyrin repeats, transcript variant 1	1.55	NM_018003
RASSF2	Ras association (RalGDS/AF-6) domain family 2, transcript variant 1	1.54	NM_014737
GPATCH1	G patch domain containing 1	1.54	NM_018025
LPAR1	Lysophosphatidic acid receptor 1	1.54	NM_057159
ARSI	Arylsulfatase family, member I	1.53	NM_001012301
TRAPPC2	Trafficking protein particle complex 2, transcript variant 1	1.53	NM_001011658
CDR1	Cerebellar degeneration-related protein 1, 34 kDa	1.53	NM_004065
WDPCP	WD repeat-containing and planar cell polarity effector	1.53	NM_015910
CCDC186	Coiled-coil domain-containing protein 186	1.52	NM_018017
RUNX2	Runt-related transcription factor 2, transcript variant 1	1.51	NM_001024630

Table 3. Downregulated DEGs in COX-2-overexpressed cell lines

Symbol	Description	Fold change	Accession No.
HIST1H4C	Histone cluster 1, H4c	4.63	NM_003542
HIST1H3H	Histone cluster 1, H3h	2.05	NM_003536
HIST1H2AI	Histone cluster 1, H2ai	1.94	NM_003509
CABYR	Calcium binding tyrosine-(Y)-phosphorylation regulated, transcript variant 1	1.91	NM_012189
FLJ16171	FLJ16171 protein	1.90	NR_046113
AKR1C2	Aldo-keto reductase family 1, member C2, transcript variant 2	1.89	NM_205845
AURKC	Aurora kinase C, transcript variant 1	1.77	NM_001015878
HIST1H1D	Histone cluster 1, H1d	1.72	NM_005320
CRLF2	Cytokine receptor-like factor 2, transcript variant 1	1.70	NM_001012288
CTAG2	Cancer/testis antigen 2, transcript variant 2	1.69	NM_020994
SERPINB5	Serpin peptidase inhibitor, clade B (ovalbumin), member 5	1.65	NM_002639
RARB	Retinoic acid receptor, beta, transcript variant 1	1.65	NM_000965
FAT4	FAT tumor suppressor homolog 4 (*Drosophila*)	1.64	NM_024582
TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	1.57	NM_002546
NOTUM	Notum pectinacetylesterase homolog (*Drosophila*)	1.57	NM_178493
FGF2	Fibroblast growth factor 2 (basic)	1.56	NM_002006
STX11	Syntaxin 11	1.53	NM_003764
VEPH1	Ventricular zone-expressed PH domain homolog 1 (zebrafish)	1.53	NM_024621
SPATA31C1	SPATA31 subfamily C, member 1	1.53	NM_001145124
ADAM21	ADAM metallopeptidase domain 21	1.52	NM_003813

DEG, differentially expressed gene; COX-2, cyclooxygenase-2.

validated (Table 2).

Functional analysis

The DEGs were concentrated in several important biological functions

The functions that were most significantly upregulated by COX-2 overexpression in U2OS cells were skeletal system development and morphogenesis, bone development and morphogenesis, ossification, and positive regulation of cell proliferation ($p < 0.05$) (Table 4). The genes of *COL1A1*, *COL5A2*, *FBN1*, *HOXD10*, *RUNX2*, and *TRAPPC2* are involved in skeletal system development and morphogenesis. The genes of *COL1A1*, *COL5A2*, and *RUNX2* are involved in bone development, morphogenesis and ossification. The genes of *DDR2*, *RAC2*, *RUNX2*, and *TSPAN31* are involved in the positive regulation of cell proliferation (Table 4).

The functions that were most significantly downregulated by COX-2 overexpression in U2OS cells were nucleosome assembly, chromatin assembly, and DNA packaging ($p < 0.05$). The genes of *HIST1H1D*, *HIST1H2AI*, *HIST1H3H*, and *HIST1H4C* are involved in these processes (Table 5).

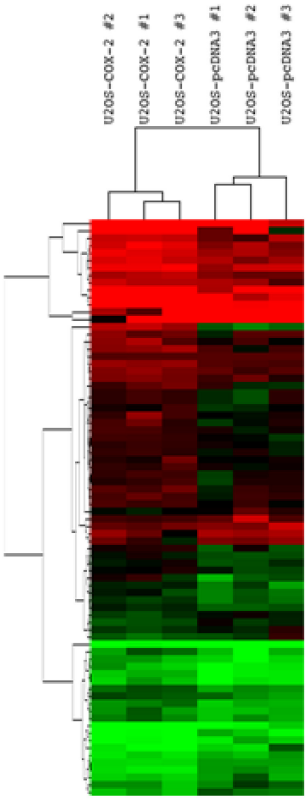

Fig. 2. Gene expression patterns of 76 differentially expressed genes (DEGs). Hierarchical cluster analysis was done between six stable cell lines using 76 DEGs. Normalized $\log_2$-gene expression values were analyzed by Cluster 3.0, and the results were visualized using Java Treeview, with red being high and green indicating low.

Discussion

COX-2 has been reported to promote cell proliferation, migration, and invasion in osteosarcoma cells. COX-2 gene knockdown by RNAi significantly reduced the cell proliferation, migration, and invasion in SaOS2 cells [11], whereas stable expression of COX-2 increased the ability in U2OS cells [14] (Table 1). These studies demonstrated that abnormal COX-2 overexpression is an important determinant for the malignant phenotype of osteosarcoma cells. By using bioinformatics tools, we here provided useful information to understand the underlying mechanism by which COX-2 promotes the malignant phenotype in osteosarcoma cells.

According to our data, the genes encoding extracellular matrix proteins are highly upregulated by COX-2 (Tables 2 and 4). The genes *COL1A1*, *COL5A2*, and *FBN1* encode $\alpha 1$ chain of type I collagen, $\alpha 2$ chain of type V collagen, and fibrillin 1, respectively. It has been known that the type I and type V collagens are major extracellular matrix proteins in

Table 4. Biological functions of upregulated DEGs

Functional category	Genes	p-value
GO_Biological process		
Skeletal system development	*PTGS2, FBN1, COL1A1, COL5A2, RUNX2, HOXD10, TRAPPC2*	0.00009
Ossification	*PTGS2, COL1A1, COL5A2, RUNX2*	0.00248
Bone development	*PTGS2, COL1A1, COL5A2, RUNX2*	0.00300
Positive regulation of cell proliferation	*PTGS2, RAC2, TSPAN31, DDR2, RUNX2*	0.01579
Endochondral ossification	*COL1A1, RUNX2*	0.02803
Skeletal system morphogenesis	*COL1A1, RUNX2, HOXD10*	0.02867
Endochondral bone morphogenesis	*COL1A1, RUNX2*	0.03720
Bone morphogenesis	*COL1A1, RUNX2*	0.04629
GO_Molecular function		
Extracellular matrix structural constituent	*FBN1, COL1A1, COL5A2*	0.01891

DEG, differentially expressed gene.

Table 5. Biological functions of downregulated DEGs

Functional category	Genes	p-value
GO_Biological process		
Nucleosome assembly	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.00005
Chromatin assembly	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.00005
Protein-DNA complex assembly	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.00006
Nucleosome organization	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.00007
DNA packaging	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.00013
Chromatin assembly or disassembly	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.00017
Cellular macromolecular complex assembly	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.00242
Cellular macromolecular complex subunit organization	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.00336
Chromatin organization	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.00395
Chromosome organization	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.00791
Macromolecular complex assembly	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.01865
Macromolecular complex subunit organization	*HIST1H1D, HIST1H2AI, HIST1H4C, HIST1H3H*	0.02219
KEGG_Pathway		
Systemic lupus erythematosus	*HIST1H2AI, HIST1H4C, HIST1H3H*	0.01236

DEG, differentially expressed gene.

bone [17]. Although there is a report that type I collagen promotes cell proliferation and migration in gastric cancer cells [18], the causal relationship between the overexpression of those genes and osteosarcoma has not been investigated well. Therefore, further studies are necessary to elucidate whether overexpression of these genes might be involved in the COX-2-mediated malignant phenotype in osteosarcoma.

Extracellular matrix proteins not only are structural components of the connective tissue in the body but also regulate cell fates through their receptors on the cell surface. DDR2 is one of the receptors for collagen, the expression of which was highly upregulated by COX-2 (Tables 2 and 4). DDR2 is a receptor tyrosine kinase, and its overexpression promotes the migration and invasion of cancer cells derived from breast, head and neck, and prostate [19-21]. Therefore, COX-2 might promote cell proliferation and motility through upregulation of DDR2. Further studies are necessary to elucidate this possibility.

RAC2, a highly upregulated gene by COX-2, is a member of the Rho family of GTPases (Tables 2 and 4). Although there is no report on Rac2 function in osteosarcoma, there is a report that Rac2 GTPase is required for B cell proliferation and survival [22]. Therefore, COX-2 might promote cell proliferation through upregulation of Rac2 function.

Among the highly upregulated genes, *RUNX2* encodes a transcription factor governing osteoblast differentiation and skeletal morphogenesis. Recent studies on Runx2 function in cancer are controversial. Runx2 overexpression reduces cell proliferation in osteosarcoma cell lines [23] but promotes cell motility or invasion in MCF-7 breast cancer cells and PC prostate cancer cells [24, 25]. Therefore, further studies are required to elucidate the function of Runx2 in osteosarcoma and the relation between COX-2 and Runx2.

Intriguingly, the genes encoding histone proteins are highly downregulated by COX-2 (Tables 3 and 5). The genes *HIST1H1D, HIST1H2AI, HIST1H3H,* and *HIST1H4C* encode histone H1H1, histone H2A1, histone H3A1, and histone H41, respectively. As is well known, the nucleosome is the basic unit of DNA packaging, consisting of a core particle and linker DNA. A core particle comprises 147 base pairs of DNA wrapped around a histone octamer (2 copies each of histone H2A, H2B, H3, and H4). The core particles are connected by 10–90 bp of linker DNA, which could be naked or bound to histone H1 [26]. The nucleosome itself has been known to be a general gene repressor [27, 28]. In addition, RNAi-mediated knockdown of H2A.Z reduced cell proliferation in bladder cancer cells [29]. These results are compatible with our finding that COX-2 downregulates the expression of histone genes. Therefore, it is worth studying the mechanism of action of histones in osteosarcoma and the mechanism by which COX-2 downregulates histone genes.

Osteosarcoma is a highly malignant tumor, and the 5-year survival rate is only 20%–30% in patients with macroscopic evidence of metastasis at diagnosis [3]. While the etiology of osteosarcoma has been largely unknown, recent studies have suggested that COX-2 plays a critical role in osteosarcoma development and progression. In this regard, we believe that our study will provide useful information to understand the mechanism of action of COX-2 in osteosarcoma.

Acknowledgments

This study was supported by 2014 Research Grant from

Kangwon National University (No. 120141490).

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