MicroRNA-650 in a copy number-variable region regulates the production of interleukin 6 in human osteosarcoma cells

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Abstract. Copy number variation is a well-known genetic variation. microRNAs (miRNAs/miRs) are non-coding RNAs that mediate gene expression by regulating target mRNAs. In the present study, copy number deletions encompassing miRNA coding regions were investigated to determine the association between the deletion of miRNA and its phenotypic effects. A total of 38 human miRNAs in copy number variants were identified and miR-650, which is functional in the human osteosarcoma MG-63 cell line, was selected. Overexpression of miR-650 decreased the expression of inhibitor of growth family member 4 (ING4) in the MG-63 cells and increased interleukin (IL)6 transcription, as well as IL6 secretion in IL1B-stimulated cells. Furthermore, miR-650 downregulated the amount of nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α and increased the transcriptional activity of nuclear factor (NF)κB. Downregulation of ING4 also increased the production of IL6, similar to miR-650 overexpression. Taken together, these data indicate that miR-650 plays a significant role in the production of IL6 by regulating ING4 expression and NFκB signaling in IL1B-stimulated MG-63 osteosarcoma cells.

Introduction

Since the completion of the Human Genome Project, gene alterations such as single-nucleotide polymorphisms (SNPs) and copy number variations (CNVs) have received particular attention in the field of disease etiology. Due to technical developments for high-throughput microarrays, sequencing and statistical methods, a number of gene alterations have been reported to be associated with diseases or phenotypic traits in genome-wide association studies (GWAS) (1). The majority of GWAS have examined the roles of SNPs in diseases, and curated resources of SNP-trait associations are available on the website for the GWAS Catalog (2). In addition to SNPs, structural alterations, such as CNVs, have emerged as another major reason for genetic susceptibility to human disease (3). SNPs and CNVs are responsible for 83.6 and 17.7% of the total number of detected genetic variations in gene expression, respectively. Extensive studies of these gene alterations may be effective for identifying the causes of human diseases and phenotypes (4).

Non-coding RNAs are functional RNA molecules that are not translated into functional proteins, but that may contribute to the regulation of a number of biological processes. microRNAs (miRNAs/miRs) are small non-coding RNAs of ~22 nucleotides in length that mediate gene silencing at the post-transcriptional level by targeting the 3'-untranslated region of the target mRNA (5). Since the initial discovery of the first miRNA, *lin-4*, from the study of post-embryonic development in *Caenorhabditis elegans*, numerous miRNAs have been identified and reported to be critical regulators of development, cellular physiology and malignancy (6-8). The majority of mammalian miRNA genes are located in defined transcription units (9). Copy number variable miRNA genes (CNV-miRNAs), which are miRNAs located in CNV regions, are potential functional variants in genotype-phenotype association studies (10).

miR-650 was identified from the human colorectal microRNAome (11), and its genomic association with the immunoglobulin (Ig) λ variable region gene was reported based on sequence comparisons and evolutionary approaches (12). Two studies have described the roles of miR-650 in gastrointestinal cancers such as gastric and colorectal cancer by regulating expression of its target genes, inhibitor of growth family member 4 (*ING4*) and N-myc downstream-regulated gene family member 2 (*NDRG2*), respectively (13,14). miR-650 is associated with several other tumors, including melanoma, lung adenocarcinoma, hepatocellular carcinoma and glioma (15-18). miR-650 expression is affected by Ig gene rearrangement and is associated with chronic lymphocytic leukemia by downregulation of its target genes,

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cyclin-dependent kinase 1, *ING4* and early B-cell factor 3 in B cells (19). *ING4*, which is a miR-650 target gene, suppresses tumorigenesis by regulating inflammatory mediators, such as interleukin (IL)6, IL8 and nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α (IκBα) (20). Furthermore, IL6 regulates the differentiation of osteoblasts and osteoclasts (21). However, no study has been published on the role of miR-650 in the production of IL6 in the human osteosarcoma MG-63 cell line.

In the present study, our previously reported CNV data that were obtained from population-based genome-wide approaches (22) was analyzed and candidate CNV-miRNAs with biological functions were identified. Additionally, the roles of miR-650 in the production of IL6, which is induced by IL1B in human MG-63 osteosarcoma cells, were investigated. The present study was approved by the Institutional Review Board of Korea Centers for Disease Control and Prevention (approval no. 2014-02EXP-10-1C-A).

Materials and methods

Identification of miRNAs overlapping with CNVs. To investigate CNV-miRNAs in the Korean population, CNV regions from our previously reported CNV study were analyzed (22). Briefly, 4,694 samples that are part of the Korean Genome Epidemiology Study were genotyped with the NimbleGen HD2 3x720 K comparative genomic hybridization array. A total of 9,388 CNV regions were identified in human genome build hg18. Among them, 3,601 CNV regions tagged by highly correlated SNPs were provided as the content for the database. In the present study, miRBase, a biological database of miRNA sequences and annotations, was used to obtain human miRNA genome coordinates with the human genome build hg19 (23). Next, the genome coordinates of hg19 were converted to those of hg18 using LiftOver in the University of California, Santa Cruz genome browser (http://genome.ucsc.edu/cgi-bin/hgLiftOver). Of the 1,872 human miRNAs in miRBase, two miRNAs, hsa-mir-1273 and hsa-mir-6724, were excluded due to the absence of genome coordinate information. Moreover, as hsa-mir-511 has two different genome coordinates, both genomic positions were included in the miRNA list. Finally, 9,388 CNV regions with 1,871 miRNA regions were compared to identify CNV-miRNAs.

Cell culture and transfection. The human osteosarcoma MG-63 cell line was purchased from the American Type Culture Collection. The medium used for routine subculture was Dulbecco's modified Eagle's medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were maintained at 37˚C in a humidified 5% CO_2 incubator. Each 20 nM of miR-650 mimic (forward, 5'-AGGAGGCAGCGCUCUCAGGAC-3' and reverse, 5'-GUC CUGAGAGCGCUGCCUCCU-3'; Bioneer, Daejeon, Korea) and *ING4* small interfering RNA (order no. 1074590; Bioneer) were transfected into MG-63 cells with Lipofectamine RNAiMAX reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequent to transfection, the cells were incubated for 48 h and then treated with 10 ng/ml IL1B (R&D Systems Inc., Minneapolis, MN, USA) prior to being harvested for further experiments.

Quantitative polymerase chain reaction (qPCR). Total RNA was isolated from cell lysates using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). Total RNA (1 μg) was mixed with the AccuPower RocketScript Cycle RT Premix (Bioneer) for cDNA synthesis according to the manufacturer's instructions. The transcribed products were used to amplify target genes. The primer sequences for PCR were as follows: miR-650 forward, 5'-AGAGGAGGCAGCGCTCT-3' and reverse, 5'-CAGTGCGTGTCGTGGAGT-3'; *ING4* (order no. P279919; Bioneer); *GAPDH* (order no. P267613; Bioneer); and *IL6* (order no. P211161; Bioneer). Amplification was performed using the Exicycler™ 96 Real-Time Quantitative PCR System (Bioneer) in a 20 μl reaction mixture containing 2 μl cDNA template (80 ng), 2.5 μl of each primer and 13 μl distilled water with AccuPower Greenstar qPCR Premix (Bioneer), including dNTP mixture. qPCR was performed under the following conditions: Initial denaturation at 95˚C for 10 min; 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec. Exicycler 3 analysis software (version 3.55.0; Bioneer) was used to calculate cycle threshold (Ct) values for all genes. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (24)

Immunoblotting analysis. Immunoblotting analysis was performed as previously described (25). Briefly, the cultured cells were rinsed with phosphate-buffered saline, scraped into 100 μl RIPA cell lysis buffer (Cell Signaling Technology Inc., Danvers, MA, USA) and placed on ice for 1 h. Next, the cells were centrifuged and the supernatant was harvested. Aliquots (20 μg) of soluble proteins were separated with SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4˚C with specific antibodies: Rabbit polyclonal antibody for IκBα and GAPDH, and goat polyclonal antibody for ING4 (1:1,000; Santa Cruz Biotechnology Inc., Dallas, TX, USA). The blot was then incubated with the corresponding horseradish peroxidase-conjugated anti-rabbit IgG or anti-goat IgG (1:5000; Cell Signaling Technology Inc.). The immune complex was visualized with enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Chalfont, UK), and image processing was performed using an image acquisition system (Fusion FX, Vilber Lourmat, Marne-la-Vallée, France).

ELISA. The culture medium of the cells that were transfected with the miR-650 mimic was collected at 6 h post-treatment with IL1B. The level of human IL6 was determined with an ELISA kit (R&D Systems Inc.).

Reporter assay. The MG-63 cells were co-transfected with the pGL4.32 [luc2p/NF-κB-RE/Hygro] containing the nuclear factor kB (NFkB) response element, with pRL-TK expressing Renilla luciferase as an internal control (Promega, USA) and miR-650 mimic with Lipofectamine RNAiMAX reagent in OPTI-MEM (Life Technologies). Following transfection, the cells were incubated for 48 h and then treated with IL1B (10 ng/ml) prior to being harvested. Cell extracts were used for the dual-luciferase assay (Promega

Table I. miRNAs located in copy number-variable regions.

ID	Chr[a]	Start	End	CNV region[b]
hsa-mir-6730	1	12561572	12561638	chr1: 12322432-12771354
hsa-mir-4256	1	112805915	112805978	chr1: 112494152-113047786
hsa-mir-4266	2	109296459	109296513	chr2: 109295476-109299710
hsa-mir-3921	3	101165848	101165932	chr3: 101111409-101181644
hsa-mir-4789	3	176570023	176570104	chr3: 176563587-176573504
hsa-mir-7978	4	21075421	21075479	chr4: 21057650-21076477
hsa-mir-1973	4	117440330	117440373	chr4: 117260800-117551180
hsa-mir-8089	5	180403009	180403090	chr5: 180362646-180406960
hsa-mir-6832	6	31709543	31709614	chr6: 31564443-31778770
hsa-mir-4646	6	31776785	31776847	chr6: 31564443-31778770
hsa-mir-3135b	6	32825667	32825734	chr6: 32734545-32828543
hsa-mir-550a-3	7	29686875	29686969	chr7: 29652945-29755706
hsa-mir-4650-2	7	71800810	71800885	chr7: 71635398-71956108
hsa-mir-3674	8	1736698	1736765	chr8: 1733768-1765533
hsa-mir-596	8	1752804	1752880	chr8: 1733768-1765533
hsa-mir-876	9	28853624	28853704	chr9: 28717924-28857119
hsa-mir-4675	10	20880905	20880981	chr10: 20871043-20896483
hsa-mir-4678	10	89253618	89253691	chr10: 89251878-89266538
hsa-mir-3166	11	87549318	87549409	chr11: 87543703-87562550
hsa-mir-6763	12	131668656	131668720	chr12: 131650394-131675091
hsa-mir-1233-1	15	32461562	32461643	chr 15: 32447266-32664650
hsa-mir-1233-2	15	32607783	32607864	chr 15: 32447266-32664651
hsa-mir-6862-1	16	28309804	28309873	chr16: 28283100-28334047
hsa-mir-6862-2	16	28643074	28643143	chr16: 28587048-28670623
hsa-mir-132	17	1899952	1900052	chr17: 1804068-1910573
hsa-mir-212	17	1900315	1900424	chr17: 1804068-1910573
hsa-mir-6129	17	44720707	44720815	chr17: 44706971-44732961
hsa-mir-4524b	17	64607278	64607392	chr17: 64601231-64619802
hsa-mir-4524a	17	64607300	64607368	chr17: 64601231-64619802
hsa-mir-4745	19	755940	756001	chr19: 726335-763296
hsa-mir-1270-1	19	20371080	20371162	chr19: 20367897-20508581
hsa-mir-1270-2	19	20371080	20371162	chr19: 20367897-20508581
hsa-mir-4752	19	59477776	59477847	chr19: 59477286-59499140
hsa-mir-3195	20	60073253	60073336	chr20: 60066989-60075374
hsa-mir-650	22	21495270	21495365	chr22: 21488908-21498416
hsa-mir-5571	22	21558447	21558559	chr22: 21551911-21565430
hsa-mir-6817	22	24181613	24181678	chr22: 24034085-24258990
hsa-mir-6818	22	28733038	28733102	chr22: 28666644-28735357

[a]Chr, chromosome (chr) number; [b]CNV region, minimal overlapping region of each copy number variation (CNV). miR/miRNA, microRNA.

Corporation, Madison, WI, USA). Firefly luciferase activity as a reporter was normalized to Renilla activity to control for transfection efficiency.

Statistical analysis. Experimental results were analyzed using R software (version 3.0.2; http://www.r-project.org/). Statistical analysis was performed with a one-way analysis of variance, and data are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of miRNAs in CNV regions and selection of miR-650 for functional analysis. From the comparative analysis of 9,388 CNV regions and 1,871 miRNA regions, 38 miRNAs on CNV regions were identified (Table I). Table I shows the genome coordinates of these 38 miRNAs. When several CNV regions with different break-points (i.e., start and end positions) encompassed the same miRNA, minimal overlapping regions between CNVs were used. Among the 38 miRNAs, the

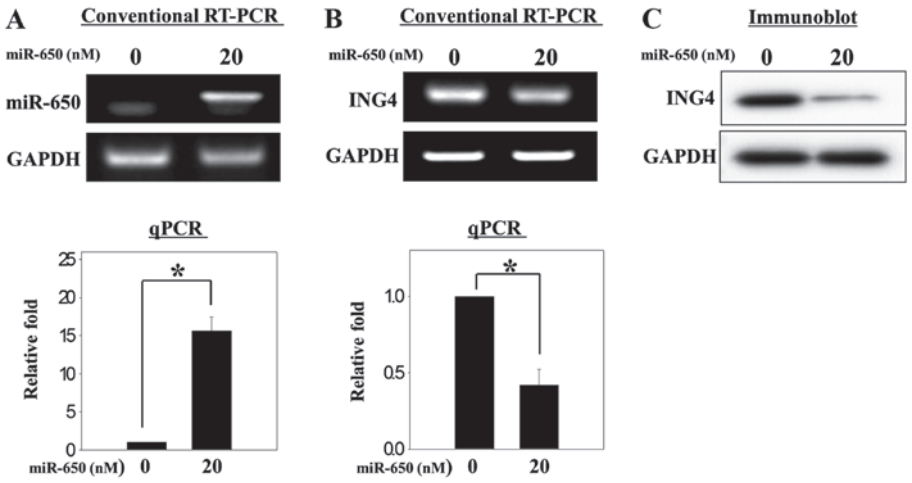

Figure 1. Overexpression of miR-650 decreases the expression of ING4 mRNA and protein in MG-63 osteosarcoma cells. (A) A total of $2.0x10^5$ cells were plated in 6-well plates and transfected with miR-650 mimic (20 nM) to overexpress miR-650. (A) miR-650 and (B) ING4 mRNA were each measured with conventional reverse transcription polymerase chain reaction (RT-PCR) and quantitative (q)PCR. (C) Protein analysis of ING4 was performed with immunoblotting. The mRNA and protein expression of GAPDH was used as a loading control. Significant differences ($^*P<0.05$) compared with the control were calculated with an analysis of variance. miR/miRNA, microRNA; ING4, inhibitor of growth family member 4.

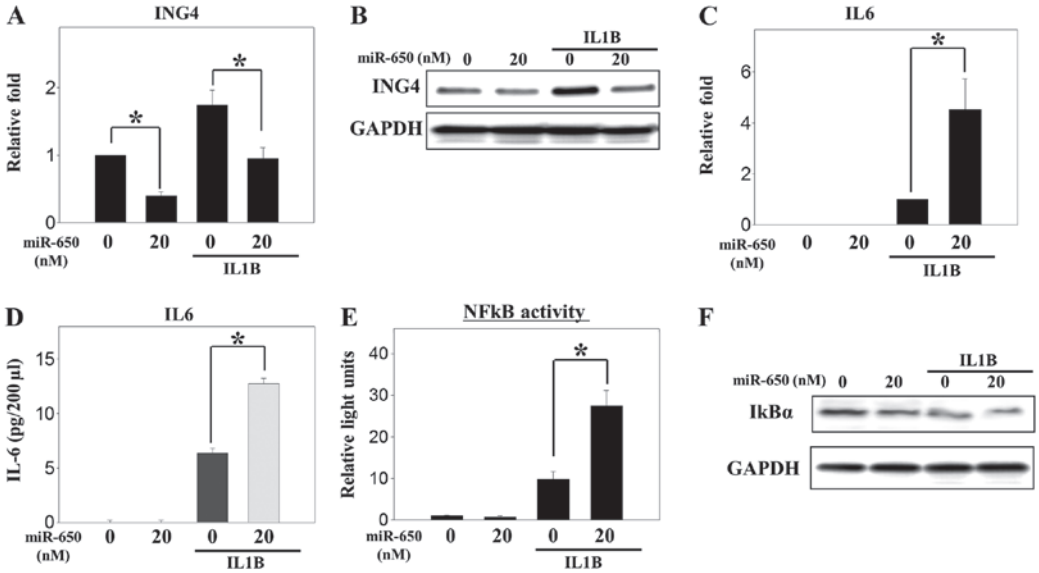

Figure 2. Overexpression of miR-650 decreases ING4 expression and increases IL6 production induced by treatment with IL1B in MG-63 osteosarcoma cells. Following transfection with the miR-650 mimic, the cells were incubated for 48 h and treated with IL1B (10 ng/ml) to induce IL6 production. (A) mRNA and (B) protein expression of ING4 and (C) mRNA expression of IL6 were detected as shown in Fig. 1. (D) Culture medium was collected and used to measure IL6 production with an ELISA. (E and F) A total of $2.0x10^5$ cells were transfected with 200 ng pGL4.32 (luc2p/NF-κB-RE/Hygro) vector and the miR-650 mimic (20 nM). After 1 h, the cells were treated with IL1B (10 ng/ml). Firefly luciferase activity, as a reporter of NFκB activity, was normalized to Renilla activity, as a control for transfection efficiency. Protein expression of IκBα was analyzed with immunoblotting. Significant differences ($^*P<0.05$) compared with the control were calculated with an analysis of variance. ING4, inhibitor of growth family member 4; IL6, interleukin 6; NFκB, nuclear factor κB; IκBα, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α.

target genes of three (miR-650, miR-132 and miR-212) have previously been reported (13,26). From those three miRNAs, miR-650 was selected for this functional study, as its location overlapped with the exon region of another transcript, and as its target genes have been identified in several cancer cell types, but not in osteosarcoma cells (14,16,17,20,27).

Overexpression of miR-650 downregulates ING4 mRNA and protein expression in MG-63 osteosarcoma cells. Although *ING4* is a target gene of miR-650 in other cancer cell types (13,17,20), it had not been evaluated in osteosarcoma cells. Thus, a miR-650 mimic was transfected to overexpress miR-650 in the MG-63 osteosarcoma cells (Fig. 1A). Overexpression of miR-650 decreased the expression of ING4 mRNA and protein (Fig. 1B and C). As MG-63 cells are osteoblast-like osteosarcoma cells, stimulation with IL1B may accelerate the production of inflammatory cytokines, such as tumor necrosis factor-α and IL6, through the NFκB signaling pathway (28). In the present study, the overexpression of miR-650 decreased the upregulation of *ING4* mRNA expression that was induced by IL1B (Fig. 2A). A similar expression pattern was observed for protein levels (Fig. 2B). These results indicate that *ING4* may

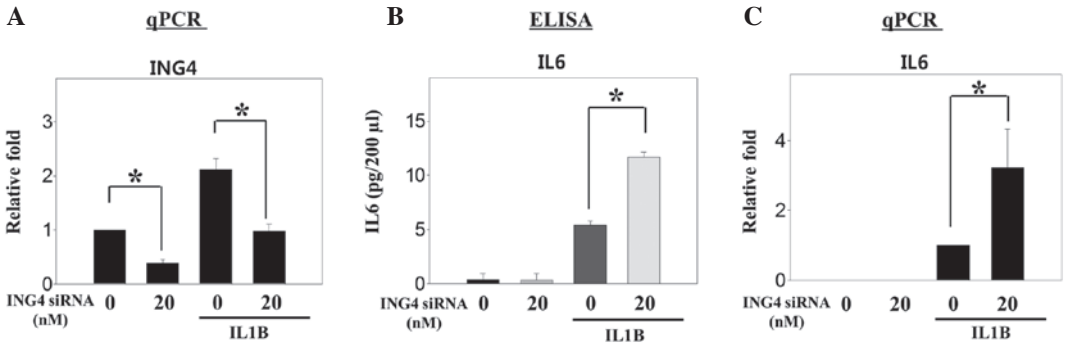

Figure 3. Knockdown of ING4 expression increases the production of IL6 that is induced by treatment with IL1B in MG-63 cells. A total of $2.0x10^5$ cells were plated and transfected with small interfering (si)RNA for ING4 (20 nM). After 48 h, the cells were harvested. (A and C) mRNA expression and (B) protein expression were analyzed with quantitative polymerase chain reaction (qPCR) and ELISA, respectively. Significant differences ($^*P<0.05$) compared with the control were calculated with an analysis of variance. ING4, inhibitor of growth family member 4; IL6, interleukin 6.

be a target gene of miR-650 in osteosarcoma cells, similar to previous results in other cancer cells.

Overexpression of miR-650 increases the production of IL6 induced by treatment with IL1B in MG-63 osteosarcoma cells. Overexpression of ING4 decreases the expression of IL6 in human umbilical vein endothelial cells (29). Therefore, we investigated the role of miR-650 in the production of IL6 induced by IL1B in MG-63 cells. As expected, IL6 mRNA and protein expression was increased by treatment with IL1B in the MG-63 cells and was more highly increased in the miR-650-overexpressing cells than in the control cells (Fig. 2C and D). ING4 may regulate the expression of IL6 by modulating NFkB activity, as reported previously in melanoma and brain tumors (29,30). Therefore, the present study investigated whether NFκB transcriptional activity is involved in the production of IL6 by miR-650 in MG-63 cells. NFκB transcriptional activity was increased by IL1B treatment and more highly increased in the miR-650-overexpressing cells than the control cells (Fig. 2E). Furthermore, IκBα protein levels were decreased by overexpression of miR-650 (Fig. 2F). These results indicate that miR-650 regulates the production of IL6 that is induced by IL1B by modulating ING4 expression and NFκB transcriptional activity in osteosarcoma cells. This is similar to the results found in other cells, such as brain tumor cells and melanoma cells (29,30).

Knockdown of ING4 expression increases the production of IL6. The present study investigated whether the effect of miR-650 on the production of IL6 induced by IL1B was mediated by ING4. The production of IL6 mRNA and protein was more highly increased in the cells in which ING4 was knocked down compared with the negative control group (Fig. 3). These results indicate that miR-650 regulates the production of IL6 by modulating its target gene, *ING4*.

Discussion

Extensive CNV studies have shown that various human diseases, including autism, schizophrenia, epilepsy, Parkinson's disease, Alzheimer's disease, chronic pancreatitis and Crohn's disease, are associated with genomic alterations (31). miRNA signatures in a number of diseases, such as melanoma and colorectal cancer, indicate that miRNAs have significant functions in human disease through the regulation of their target genes (11,15). Gene alterations such as CNVs and SNPs in miRNA genes in the human genome are potential variants for studying the functional roles of miRNAs in human disorders (10). In the present study, 38 CNV-miRNAs were identified from the CNV discovery study of a Korean cohort using the NimbleGen HD2 3x720 K comparative genomic hybridization array. miR-650 was selected for further studies and its role was analyzed in the production of the inflammatory cytokine, IL6, in MG-63 osteosarcoma cells.

miR-650 was selected for further functional studies, as its target genes, *ING4* and *NDRG2*, are important in gastric and colorectal cancer cells, respectively (13,14). In addition, miR-650 has previously been identified as a CNV-miRNA in another study (10). ING4 is a member of the ING family that acts as a tumor suppressor protein and is a promising candidate for the development of novel therapies in cancer research (13,17,29,30,32). In the present study, the overexpression of miR-650 decreased the expression of ING4 mRNA and protein levels (Fig. 1), and decreased the expression of the ING4 that was upregulated by treatment with IL1B to induce IL6 (Fig. 2).

A number of studies have described a role for ING4 in tumorigenesis and innate immunity by regulating the expression of p53, tumor necrosis factor α, IL6, IL8, matrix metalloproteinases, cycloxygenase-2 and IκBα. Among the genes regulated by ING4, *IL6* encodes a multifunctional cytokine that activates target genes involved in a wide range of biological activities (20,27,30,33). IL6 also modulates osteoblast and osteoclast differentiation. Recently, IL6 in osteosarcoma cells was reported to promote the expression of intercellular adhesion molecule-1 and cell motility (21,34). Although roles for ING4 and IL6 in tumorigenesis and inflammation have been reported, no study has described the role of miR-650 in the production of IL6 in osteosarcoma cells. Based on previous studies, we speculated that the regulation of IL6 by ING4 may be due to the activity of miR-650. As expected, miR-650 increased the production of IL6 that was induced by IL1B by downregulating ING4 in the MG-63 osteosarcoma cells (Fig. 2).

ING4 regulates IL6 production by modulating NFκB activity (29). Therefore, the present study investigated the modulation of NFκB by miR-650 using an NFκB reporter assay, and measured the amount of IκBα protein. As shown in Fig. 2E and F, the overexpression of miR-650 increased the activity of NFκB transcriptional activity and regulated the amount of IκBα protein. Regulation of NFκB activity by ING4 may be due to a physical interaction between ING4 and p65 (RelA), a subunit of NFκB, that results in decreased activation of the canonical NFκB-responsive promoter in brain tumor cells (30). A previous study showed that ING4 promotes IκB promoter activation to suppress NFκB signaling (35). The present study did not investigate whether ING4 directly interacted with p65 or how ING4 regulates the NFκB signaling pathway to induce the differential expression of NFκB target genes. However, it may be concluded that miR-650 regulates IL6 production by modulating ING4 expression and subsequent NFκB signaling in osteosarcoma cells. Therefore, this study may be the first to elucidate the role of miR-650 in the production of IL6 in IL1B-stimulated osteosarcoma cells.

Taken together, these data indicate that the overexpression of miR-650 increases the production of IL6 induced by IL1B treatment in MG-63 osteosarcoma cells by regulating ING4 expression and subsequent NFκB transcriptional activity. Additionally, this study suggests that miR-650 may be an upstream regulator of IL6 production in MG-63 osteosarcoma cells and could be a candidate therapeutic target for IL6-related human diseases, including cancer.

Acknowledgements

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