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Polycomb group molecule PHC3 regulates polycomb complex composition and prognosis of osteosarcoma

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Polyhomeotic homolog 3 (PHC 3) is a member of the human polycomb complex and has been regarded as a candidate tumor suppressor of osteosarcoma. In the present paper, we performed a mutation survey and PHC3 expression analysis by quantitative real-time PCR using 10 osteosarcoma cell lines and 42 primary osteosarcoma samples. Relative PHC3 expression values of clinical samples were analyzed with clinical outcomes, and it was suggested that lower PHC3-expressing patients had significantly worse overall survival. Relative PHC3 values of clinical samples were less than those of normal bone tissues, whereas they were greater than those of cell lines. By denaturing high performance liquid chromatography analysis and direct sequencing, we found a PHC3 missense mutation in U2OS cells, which resulted in arginine56 to proline substitution. The same point mutation existed in four of 42 primary osteosarcoma samples. Regarding functional analysis, PHC3 expression significantly suppressed the colony formation of tumor cells. Intriguingly, polycomb repressive complex 1 members, Bmi1 and Ring1b proteins, were reduced in PHC3-expressing osteosarcoma cells. Deletion mutant PHC3 expression suggested that the carboxyl terminus of PHC3 has a role in suppression; the above-mentioned point mutation of PHC3 also lost inhibitory activities. Conversely, Bmi1 expression reduced PHC3 at the mRNA level and induced the proliferation of osteosarcoma cells. Taken together, we confirmed the role of PHC3 as a tumor suppressor in osteosarcoma cells and found that PHC3-dependent tumor suppression may be caused by modification of the composition of polycomb repressive complex 1 in cancer cells. (*Cancer Sci* 2010; 101: 1646–1652)

Tumorigenesis is an aberrant differentiation in cells that involves the inappropriate regulation of developmental genes and cellular signaling pathways leading to tumor onset. Besides the already-known genetic changes that occur in cancer, such as the amplification/mutation of oncogenes, deletion/mutation of tumor suppressor genes, and loss of heterozygosity, epigenetic lesions such as altered genomic DNA methylation, aberrant regulation of chromatin remodeling by histone modifications, dysregulated expression of polycomb group (PcG), and trithorax group (TrxG) proteins have emerged as common hallmarks of many tumors.[1,2] PcG proteins are epigenetic gene silencers that are implicated in neoplastic development. Their oncogenic function might be associated with their well-established role in the maintenance of embryonic and adult stem cells. Components of polycomb repressive complex 1 (PRC1) (such as Bmi1[3]) and PRC2 (such as EZH2: enhancer of zeste homolog 2[4]) are amplified and/or overexpressed in a broad spectrum of tumors, suggesting the roles of polycomb group proteins as oncogenes in several tumors. Importantly, dysregulation in the expression of PRC components is associated with alterations in PcG protein compositions[5] as well as with target gene affinities.[6]

Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents.[7] Despite recent progress in combined therapeutic modalities, the survival of advanced stage patients is still poor. For patients with metastatic disease at initial presentation, about 20% will remain continuously free of disease, and roughly 30% will survive for 5 years from diagnosis.[8] To improve the prognosis of advanced stage patients, the development of new molecular target treatments based on the identification of key molecules for OS tumorigenesis will be required. Consecutive investigation for searching potential molecular targets of OS treatment has been performed for years, and many genes have come up as candidates of tumor-related genes of OS, such as Rb and p53; p16INK4a (INK4: inhibitor of CDK4) and p14ARF (alternative reading frame); MDM2 (transformed mouse 3T3 cell double minute 2) and CDK4 (Cyclin-dependent kinase 4); and ERBB2, c-MYC, and FOS/JUN.[9] The result of several clinical investigations, however, indicated to us that further study of tumor-related genes would be required to develop molecular-targeted therapy for OS. Therefore, we focused on the analysis of the functional roles of PHC3 in OS cells because PHC3 is mapped to 3q26, which has been regarded as a tumor-specific LOH locus in osteosarcoma,[10] and a report has indicated that several nonsense mutations of PHC3, a member of PRC1 complex, were found in OS tumor samples, and immunohistochemical analysis showed loss of expression in 64% of tumor samples.[11] However, the epigenetic changes of OS cells have not been studied extensively.

In the present study, we found that PHC3 is a prognostic factor for OS patients treated with the standard therapeutic modalities. PHC3 expression in tumor cells, including OS cells, resulted in decreased colony formation; carboxyl terminal deletion recovered impaired colony formation ability. Furthermore, a missense mutant detected in U2OS cells was found in four of 42 OS tumor samples and the mutant also lost ability as a tumor suppressor.

Materials and Methods

Tumor specimens and patient information. Tumor samples were obtained from 42 patients (25 males and 17 females; median age, 17 years) who had been treated at Chiba Cancer Center (Chiba, Japan). Three normal bone tissues were also obtained from benign bone tumor patients (separated from the lesion). All samples were obtained with appropriate informed consent and investigated according to protocols approved by the institutional review board. Forty samples were diagnosed as conventional OS and two were high-grade surface OS, according to the World Health Organization histological classification of bone

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Table 1. Characteristics of the 42 osteosarcoma patients and relation to overall survival

Characteristics	No.	5-year OAS (%)†	*P*-value*
Sex			
Male	25	65.0	0.15
Female	17	82.0	
Age at diagnosis			
≤20 years	30	81.2	0.58
>20 years	12	67.5	
Site			
Extremities	37	78.7	0.89
Trunk	5	66.7	
Stage			
Stage 2A	15		
Stage 2B	19	79.9	
Stage 3	4		0.009‡
Stage 4A	4	0	
Relative *PHC3* expression level			
>0.253	21	87.9	0.03
≤0.253	21	61.5	

*Significance was estimated using the log-rank test. †5-year overall survival. ‡Stage 2A+2B+3 *versus* Stage 4A.

tumors.[12] The clinical stages of the disease were determined at the time of initial biopsy according to the TNM classification system for malignant tumors defined by the International Union Against Cancer.[13] All OS patients had definitive surgery as local therapy and common neoadjuvant chemotherapy, with high-dose methotrexate, cisplatin, doxorubicin, and ifosfamide according to NECO-95J, Japanese multi-institutional phase II study.[14] A summary of the clinical records and pertinent characteristics is presented in Table 1.

DNA/RNA extraction and semi-quantitative RT-PCR. Total RNA was extracted from OS cell lines, frozen primary OS tumor tissues, and normal bone tissues using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Genomic DNA was isolated from cell lines and frozen tumor samples by a previously described method.[15] Methods of semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis were described previously.[15] Briefly, cDNA was synthesized from 1 μg total RNA templates according to the manufacturer's protocol (RiverTra-Ace-α RT-PCR kit; Toyobo, Osaka, Japan). Primer sequences are described in Table S1.

Quantitative real-time PCR. Quantitative real-time PCR (RQ-PCR) analysis was performed using the ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions using SYBR Premix Dimer Eraser (Takara Bio, Shiga, Japan). GAPDH was used as an endogenous control. The primer sequences are described in Table S1. Each sample was amplified and analyzed in triplicate.

Cell culture. Human OS cell lines U2OS, SaOS2, SJSA1 (obtained from the American Type Culture Collection, Manassas, VA, USA), HuO3N1, HuO9N2 (Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan), NY, HuO9, G292 (Health Science Research Resources Bank, Osaka, Japan), HOS, and MG63, as well as MCF7 human breast carcinoma cells (RIKEN Bioresource Center, Tsukuba, Japan), were cultured in DMEM or RPMI (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 50 μg/mL penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in an incubator with humidified air at 37°C with 5% CO_2.

Western blot analysis. Protein extraction and western blot analysis were performed as reported previously.[15] After transferring to an Immobilon-P membrane (Millipore, Billerica, MA, USA), proteins were reacted with either anti-PHC3 rabbit polyclonal (A301-569A; Bethyl Laboratories, Montgomery, TX, USA), anti-Bmi1 mouse monoclonal (229F6; Upstate, Lake Placid, NY, USA), anti-β-Actin rabbit polyclonal (Sigma, St. Louis, MO, USA) or anti-tubulin mouse monoclonal (Lab Vision, Fremont, CA, USA) antibodies. Anti-Ring1b mouse monoclonal antibodies were as described in a previous report.[16]

Plasmids and transient transfection. FLAG-tagged PHC3 expression plasmid, FLAG-PHC3-pcDNA3, containing the entire coding region of human *PHC3* (RefSeq NM_024947.3), was constructed into the expression plasmid pcDNA3. For overexpression of Bmi1, FLAG-tagged Bmi1 plasmid was used.[17] A series of carboxyl terminal-deleted constructs (Del.1 [deleted sterile alpha motif, SAM, domain], Del.2 [deleted SAM and four conserved cystenes, FCS, domains], Del.3 [deleted SAM, FCS, and homology domain I, HD1, domains]), as well as a point mutant PHC3 G201C construct, were derived from the FLAG-PHC3-pcDNA3 plasmid. Empty plasmid pcDNA3 was used as a transfection control. Transient transfection was performed using FuGENE HD Transfection Reagent (Roche Molecular Biochemicals, IN, USA) according to the manufacturer's protocol. We collected cells 48 h after transfection and the cells were subjected to the following experiments.

Cell proliferation assay. Cells were seeded in 96-well plates at a density of 10^3 cells/well in a final volume of 100 μL. The culture was maintained in 5% CO_2, 10 μL WST-8 labeling solution (Cell counting Kit-8; Dojindo, Kumamoto, Japan) was added, and the cells were returned to the incubator for 2 h. The absorbance of the formazan product formed was detected at 450 nm in a 96-well spectrophotometric plate reader, as per the manufacturer's protocol.

Colony formation assay. Twenty-four hours after transfection, 1×10^4 cells were seeded into 6-cm diameter dishes and selected by 500 μg/mL G418 (Gibco BRL, Grand Island, NY, USA). Media with G418 was changed every 3 days. The cells were then fixed and stained with Giemsa, and the number of colonies was scored.

Mutation analysis. Mutation analysis of *PHC3* was performed using DNA extracted from 10 OS cell lines. All exons (exon 1–15) and intron-exon boundaries of *PHC3* were amplified using genome-specific intronic primers. The primers for this analysis are indicated in Table S2. Direct sequencing of the amplified fragments was performed using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Wellesley, MA, USA) and analyzed by an ABI Prism 3900 DNA Analyzer automated sequencer (Applied Biosystems). Subsequently, a single nucleotide substitution, G201C, found in U2OS was further investigated by denaturing high performance liquid chromatography (WAVE System; MD Transgenomic, Omaha, NE, USA) using DNA extracted from 42 frozen OS samples.

Statistical analysis. Patients were grouped according to various clinical aspects of disease. The significance of differences in the characteristics between patient groups was examined using Student's *t*-test. Overall survival of each group of patients was estimated using the Kaplan–Meier method, and compared using the log-rank test. Differences between the two populations were judged significant at confidence levels >95% ($P < 0.05$).

Results

Low expression level of PHC3 in OS samples is correlated with unfavorable outcome. Total RNA was extracted from 42 primary OS samples treated at Chiba Cancer Center from 1996 to 2007. Of the 42 patients, 31 were alive and 11 were dead at the

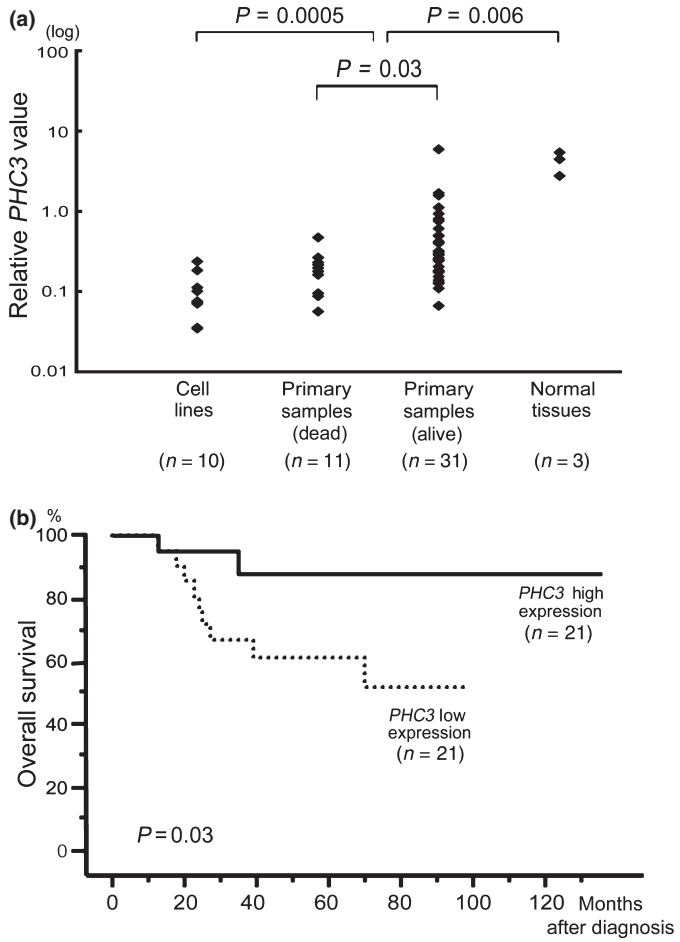

Fig. 1. Quantification of PHC3 expression in osteosarcoma (OS) cell lines and primary samples. (a) PHC3 expression in OS cell lines, primary OS samples, and normal bone samples. *PHC3* mRNA expression was determined by RQ-PCR and normalized by *GAPDH* values. Values are presented on a logarithmic scale. (b) Kaplan–Meier survival analysis of OS patients based on higher or lower expression levels of *PHC3*. Curves show that the overall survival rate was significantly lower in low PHC3-expressing patients. Statistical analysis was performed by log-rank test. Corresponding *P*-values are indicated.

time of this research. The relative expression of *PHC3* ranged from 0.0563 to 5.934 (median, 0.253). Compared to the *PHC3* expression of normal bone tissues and OS cell lines, primary OS tissue showed a significantly lower *PHC3* expression than normal bone tissues but higher than the cell lines (Fig. 1a). Moreover, the poor outcome cohort showed a significant reduction of PHC3 expression.

Next, we divided 42 OS cases into two groups based on each PHC3 expression level (PHC3 high expression group [>0.253], $n = 21$ and PHC3 low expression group [≤0.253], $n = 21$). Kaplan–Meier survival analysis showed that each clinicopathological feature except tumor stage did not show a significant difference in 5-year overall survival rate (Table 1); however, the 5-year overall survival rate was significantly lower in PHC3 low expression OS cases ($P = 0.03$ by log-rank test; Fig. 1b). These results indicated that the relative PHC3 expression level, as with the tumor stage, which is a well-known prognostic factor of OS, seems to be a prognostic factor of OS. Since previous paper indicated that PHC3 expression was suppressed at protein level in OS tumor samples,[11] we also tried immunohistochemichal analysis of OS samples (Fig. S1). However, there were not enough paraffin-embedded OS samples to confirm the statistical significance of PHC3 expression in prognosis of OS patients.

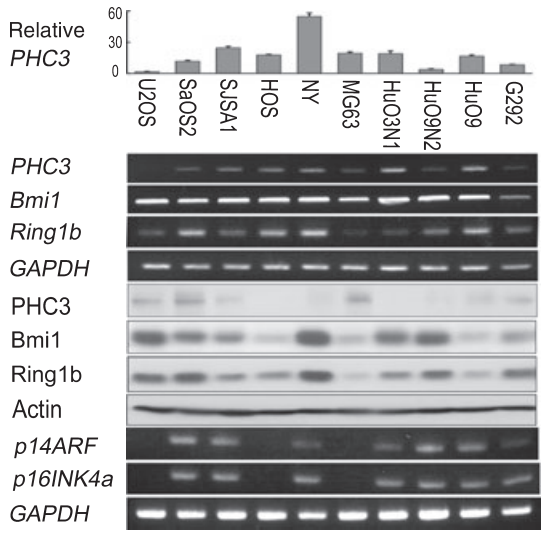

Fig. 2. Expression profile of polycomb groups (PcGs) and p14/16 in osteosarcoma (OS) cell lines. Expression level of PHC3 was analyzed by RQ-PCR, semi-quantitative RT-PCR, and western blotting. In quantitative real-time PCR (RQ-PCR), relative *PHC3* values were normalized by *GAPDH*. Data are representative results of at least three independent experiments.

PHC3 expression is not correlated with those of other PcG genes in OS cell lines. We analyzed mRNA and protein levels of *PHC3*, as well as other polycomb group genes, *Bmi1* and *Ring1b*, in 10 OS cell lines. These three genes are regarded as members of the same complex, PRC1. According to the results of semi-quantitative RT-PCR and RQ-PCR, each OS cell line expressed various levels of PHC3 and, interestingly, each PHC3 protein expression was not correlated to those of other polycomb group genes, although there was a tendency toward a correlation between Bmi1 and Ring1b proteins (Fig. 2). The correlation between mRNA and protein expressions of PRC1 molecules was not so obvious in OS cells (Fig. 2), suggesting the regulation of protein stabilization in the polycomb complex.

PHC3 suppresses OS cell proliferation with the reduction of Bmi1. To investigate the function of PHC3 in OS cells, we transiently expressed PHC3 in OS cells. Expression of wild-type PHC3 in HOS and G292 cells suppressed colony formation (Fig. 3a). Western blot analysis and semi-quantitative RT-PCR showed that Bmi1 and Ring1b expressions were reduced at the protein level in PHC3-expressing cells. Although *p14ARF/p16INK4a* were not expressed in HOS cells, these two PRC1 target mRNAs were increased in G292 cells by PHC3 expression.

Next, PHC3 has three highly conserved domains in the carboxyl terminal regions of all mammalian and *Drosophila* members of the polyhomeotic family, namely the sterile alpha motif (SAM), four conserved cystenes (FCS), and homology domain I (HD1).[18,19] The SAM domain has a critical role in homo-oligomerization [20] and the FCS domain is essential for RNA binding. [21] These domains may also have a role in the interaction between PRC1 and nucleosome through the protein–protein and protein–RNA bindings.[22] On the other hand, the function of HD1 is unknown. To address the role of PHC3 domains for the suppression of colony formation, we constructed three plasmids, named Del.1, Del.2, and Del.3, which express carboxyl terminal deleted PHC3 constructs from the carboxyl terminus to the SAM domain, the FCS domain, and the HD1, respectively (Fig. 3b). Ectopic expression of deletion mutant PHC3 was confirmed by western blotting at the appropriate band size of each plasmid. Del.1/Del.2 mutants suppressed colony formation and Bmi1 protein expression; however, Del.3 mutant failed to

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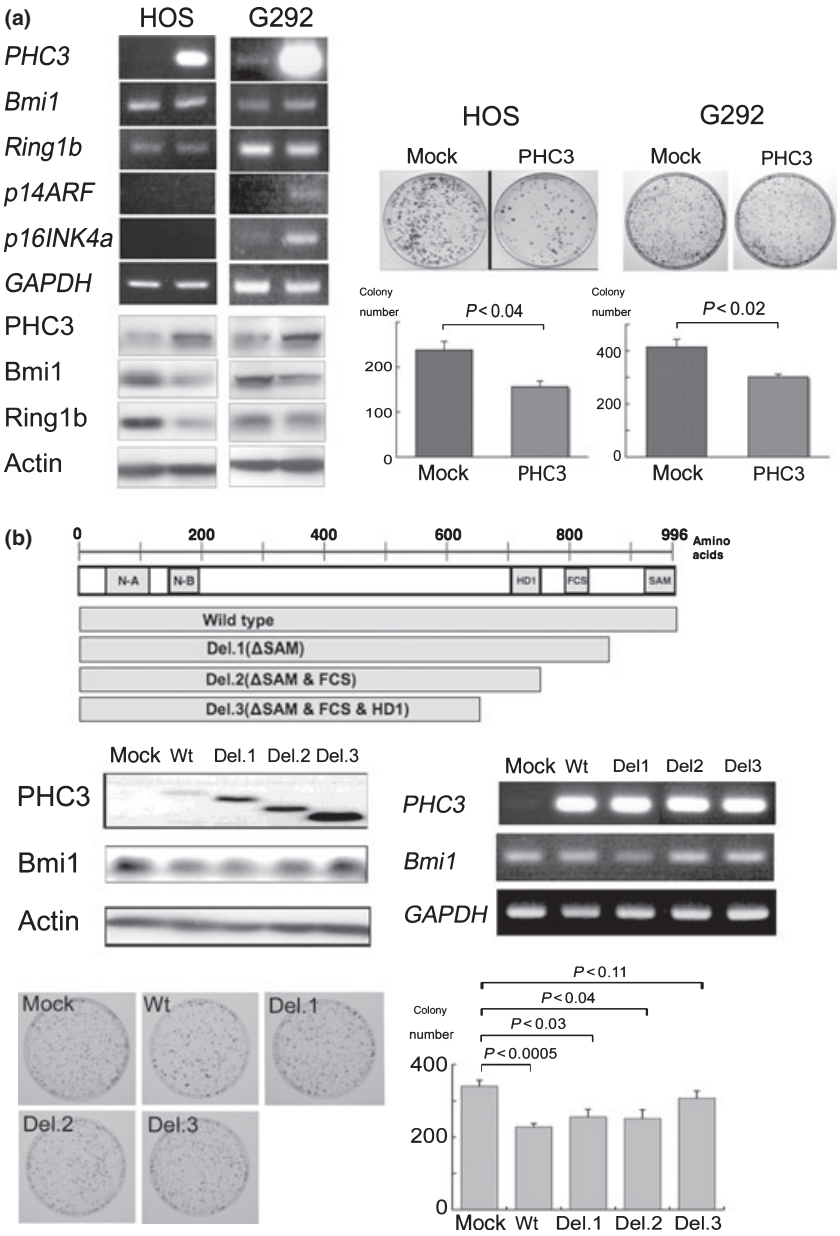

Fig. 3. Polyhomeotic homolog 3 (PHC 3) suppresses cell proliferation of osteosarcoma (OS) cells and modifies expression of polycomb repressive complex 1 (PRC1) proteins. Transient transfection of PHC3 expression plasmids, semi-quantitative RT-PCR, and western blotting were performed as described in the Materials and Methods. Cell proliferation was studied by colony formation assay. Data are representative results of at least three independent experiments. (a) Effect of PHC3 over-expression. HOS and G292 cells were used for experiments. (b) Deletion mutant PHC3-related effects on OS cells. Structures of mutants are indicated by schematic drawing. Three deletion mutants of PHC3 expression plasmids were transfected into G292 cells and analyzed by western blotting, semi-quantitative RT-PCR, and colony formation assay.

repress Bmi1 and colony formation. RT-PCR analysis indicated that Bmi1 regulation was at the protein level.

Mutant form PHC3 loses its tumor suppressive function. We examined all 15 exons of the *PHC3* gene in 10 OS cell lines to detect mutation. This direct sequencing identified a single nucleotide substitution, G201C, which results in a missense mutation converted from arginine56 to proline in only U2OS cells (Fig. 4a). Subsequently, we performed a mutation search by the WAVE system to elucidate whether this missense mutation exists in 42 OS primary samples (Fig. 4b). Four samples (9.5%) retained the same mutation pattern in WAVE analysis. Direct sequencing confirmed these mutations (Fig. 4c). Two of these patients were alive and free from disease 77 and 99 months after treatment. Regarding the remaining patients, one was alive with disease and one died of recurrence. The expression levels of PHC3 in the four mutant cases were not so low in the tumor samples. Although we tried to analyze the existence of the G201C mutation in germ line, we could not obtain patients' agreement. We studied the human SNPs database NCBI dbSNP (BUILD 130) (http://www.ncbi.nlm.nih.gov/snp) for the PHC3 G201C alteration to address the significance of the PHC3 mutation and found that there was no report of the G201C SNP in the database.

To investigate the function of this mutant PHC3, we generated the mutant form of the PHC3 expression plasmid and transiently transfected into OS G292 cells and breast cancer MCF7 cells. The results of the cell proliferation assay indicated that the mutant form of PHC3 partially (MCF7) and completely (G292) lost the ability to suppress cell proliferation as wild-type PHC3. These results were confirmed by colony formation assay (Fig. 4d). Intriguingly, Bmi1 suppression was cancelled by the mutation in G292 cells and the effect of the mutant on proliferation was marginal in MCF7 cells.

Bmi1 ectopic expression reduces PHC3 expression in OS cell lines. We next investigated the effects of Bmi1 on PHC3 expression in OS cell lines. We transfected Bmi1 expression plasmid into two OS cell lines, SaOS2 and G292. As expected, Bmi1 expression in OS cells significantly accelerated the colony

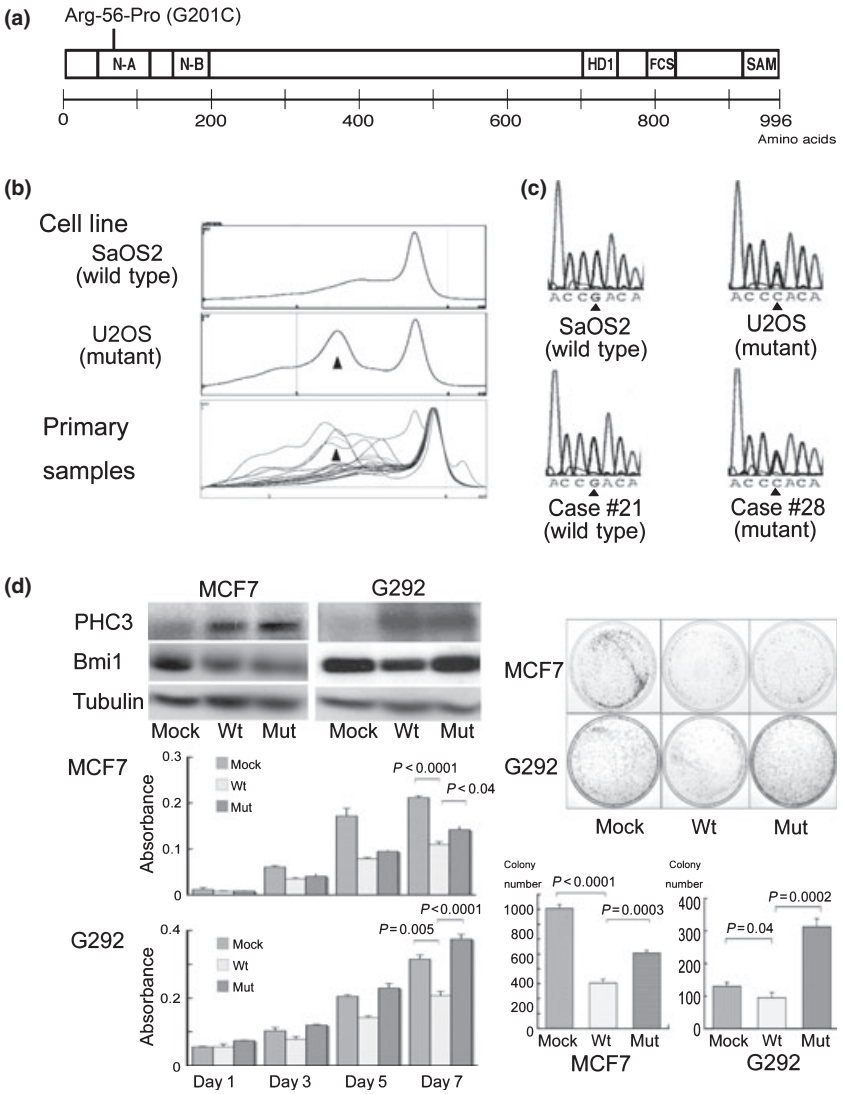

Fig. 4. A mutant form of Polyhomeotic homolog 3 (PHC 3) found in osteosarcoma (OS) loses its tumor suppressive function. (a) Schematic presentation of single nucleotide substitution of PHC3. G201C substitution resulted in arginine56 to proline mutant PHC3 (initiation methionine is coded at 35–37 of NM_024947.3). (b) Mutation screening in exon 2 of PHC3. Forty-two OS primary samples were analyzed by WAVE systems. Arrowheads indicated a mutant pattern of WAVE assay detected in U2OS cells. Similar patterns were also detected in four of 42 samples. (c) Direct sequencing results of PHC3 exon 2. Four of 42 samples (9.5%) showed G201C mutation. (d) Arg56Pro mutant PHC3 lost tumor suppressor function. Transient transfection of wild-type and Arg56Pro mutant PHC3 expression plasmids, semi-quantitative RT-PCR, and western blotting were performed as described in the Materials and Methods. Bmi1 protein expression was also analyzed. The results of the cell proliferation assay and colony formation assay are representative of at least three independent experiments.

formation of these OS cells (Fig. 5), which could be dependent on the suppression of p16INK4a transcription. Interestingly, PHC3 expression was suppressed at both the transcriptional and translational levels by Bmi1 expression (Fig. 5).

Discussion

Within the members of PcG proteins, oncogene function has been reported but not tumor suppressor, and PcGs are overexpressed in a broad spectrum of tumors.[1,2] PHC3 is the first PcG member which suggested the role of tumor suppressor in OS[11] and we studied the effect of PHC3 expression on tumor cells and confirmed that PHC3 has a role as a tumor suppressor (Figs 3,4) and also is a prognostic factor in OS patients (Fig. 1). Previously, we found that the modification of PRC1 protein amounts changes other PRC1 protein levels coincidentally.[23] In the present study, our experiments indicated that PHC3 expression seems to affect the protein amount of other PRC1 complex members, Bmi1 and Ring1b, in OS cells and modulates PRC1 function (Figs 2,3). In the previous report, it was mentioned that PHC3 interacts with other polycomb members, Bmi1, M33, and Yin Yang 1 (YYI), in adult mouse bone.[11] In the PRC1 complex, protein interaction was mediated through the RING domain (Ring1 and Bmi1) and SAM domain (Ph). Our carboxyl terminal deletion mutant of PHC3 lost SAM/FCS/HD1 domains, indicating diminished ability in the suppression of colony formation and Bmi1 amounts (Fig. 3). This suggested that PHC3 may modulate the composition of PRC1 complex and the functions of PRC1 by interacting with other PRC1 complex molecules. Given that the previously reported nonsense mutations in OS tumors resulted in deletion mutant PHC3s, these mutants seem to be loss of function PHC3 in OS.[11] We also found a point mutation of PHC3 (G201C) in the N-A domain (Fig. 4a). The G201C mutation did not affect the protein amount of PHC3 in several tumor cells, and the effect on colony formation suppression was diminished compared to wild-type PHC3 (Fig. 4d). However, further study will be required to confirm the significance of the mutation in OS since the number of analyzed patients was not so high in the present study. Another interesting observation in our study is that Bmi1 expression suppressed PHC3 transcription (Fig. 5), suggesting that PRC1 complex might repress PHC3 transcription by the original PRC1 function, for example promoter modification; however, since these experiments were performed by enforced Bmi1 expression, more physiological experiments will be required to confirm the findings.

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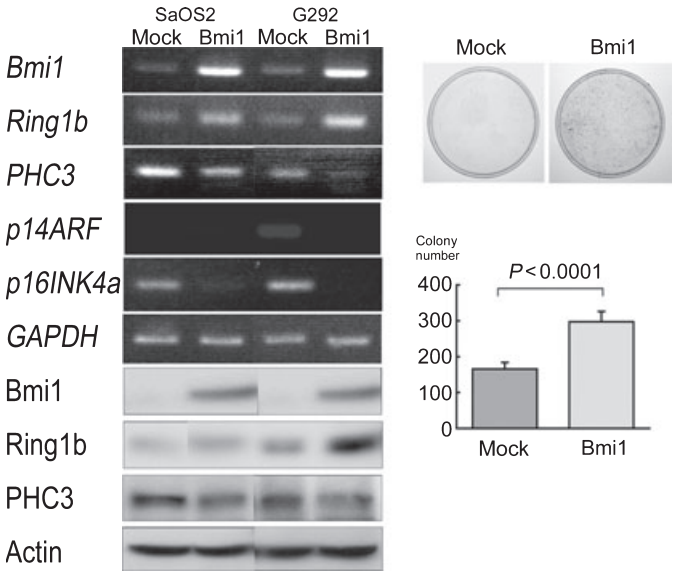

Fig. 5. Effects of Bmi1 on PHC3 expression and cell proliferation. Bmi1 expression plasmid was transiently transfected into SaOS2 and G292 cells. Semi-quantitative RT-PCR, western blot, and colony formation assay were performed as described in the Materials and Methods. The results are representative of at least three independent experiments.

The INK4A/ARF locus, localized to 9p21 region, encodes p16INK4a, a tumor suppressor that functions in part through the inhibition of CDK4 and p14ARF, and another tumor suppressor regulates p53 function by inactivating MDM2 by nucleolus sequestration.[24] INK4A/ARF is a well-known target locus for polycomb-mediated suppression of transcription in cancer cells.[25] In 87 OS specimens from 79 patients, INK4A/ARF changes were observed in five of 55 cases examined (four deletions and one rearrangement), whereas no INK4a exon 2-point mutations or methylation were detected.[9,26] Although the rate of genetic change of INK4A/ARF was not so high in OS, epigenetic changes of INK4A/ARF were reported. The methylation status of the p16INK4a gene was studied in the 15 p16INK4a-negative OS tumors: eight samples showed 5′ CpG-island methylation; four of eight had a complete methylation status, while in the remaining four, the gene was only partially methylated, suggesting that epigenetic changes except for promoter methylation might have a role in 11 of 15 OS samples.[27] Benassi *et al.*[28] reported that p16INK4a and p14ARF proteins were negative or weakly detectable in 60% and 57% of OS cases, respectively. Oh *et al.*[29] reported that the promoter of p14ARF was methylated in 47% of 22 OS tumor samples and poor survival was related to the methylation of p14ARF. This may indicate that promoter methylation has a major role in p14ARF epigenetic regulation; however, explicit and detailed analysis will be required to identify the exact rate of p14ARF alteration analysis. In our study, neither p14ARF nor p16INK4a mRNAs were detected in three of 10 OS cell lines; promoter methylation of INK4A/ARF locus was reported in U2OS cells (Fig. 2). Interestingly, PHC3 expression effectively suppressed colony formation not only in p14ARF/p16INK4a-increased G292 cells but also in p14ARF/p16INK4a-diminished HOS cells (Fig. 3), suggesting that the polycomb complex may have other target tumor suppressors except for p14ARF/p16INK4a in OS cells. Further study of PHC3-related PRC1 targets in gene repression will be required.

Taken together, our study suggests the importance of PRC1 regulation in OS tumorigenesis and aggressiveness. Study of the effects of PRC1 on expression profiling and promoter modification in OS cells will be required to address the further role of polycomb complex in OS.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

OAS	overall survival
PHC3	polyhomeotic homolog 3

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. PHC3 protein expression in osteosarcoma (OS) tumor tissues.

Table S1. Primers for RT- and RQ-PCR.

Table S2. Primers for mutation analysis.

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