CDK4 overexpression is a predictive biomarker for resistance to conventional chemotherapy in patients with osteosarcoma

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Abstract. Osteosarcoma (OS) is the most common malignant bone tumor, and its sensitivity to preoperative chemotherapy is a significant prognostic factor. The present study aimed to identify potential genomic markers for the prediction of chemosensitivity in patients with OS using a genomic approach. A total of 50 pediatric and adolescent patients diagnosed with high-grade OS were selected. Each pre-therapeutic biopsy sample was subjected to comparative genomic hybridization array analysis and targeted exome sequencing. Although no recurrent gene mutation was observed in chemoresistant tumors, copy number analysis detected recurrent gain of chromosome 12q14.1, which was significantly more frequent (5/21; 24%) in the poor responder cohort than in the good responder cohort (0/29; 0%; P<0.01). Subsequent expression analysis revealed that *CDK4* was the only gene in the 12q14.1 gained region with an expression level that was positively associated with copy number gains. In order to elucidate the effect of CDK4 on drug sensitivity, CDK4-overexpressing OS cell lines were treated with cisplatin (CDDP); significant attenuation of CDDP sensitivity, demonstrated by increased cell viability and decreased expression of cleaved caspase-9, was induced by enforced expression of CDK4. In addition, treatment with CDK4/6 inhibitor palbociclib in CDK4-overexpressing U2OS cells facilitated apoptosis and a significant decrease in cell viability in a dose-dependent manner. In conclusion, the results of the present study showed that higher expression and amplification of *CDK4* in tumors is a predictive biomarker for resistance to conventional chemotherapy in patients with OS and that palbociclib is a promising drug for this therapeutically challenging cohort.

Introduction

Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents (1). The current treatment strategy for primary OS includes neoadjuvant chemotherapy and definitive surgery. A methotrexate, adriamycin and cisplatin (CDDP) (MAP) regimen is administered as a standard neoadjuvant treatment. Despite this multimodal approach, ~30% of patients with OS die due to relapse (2,3); this mortality rate has not changed for >20 years.

Sensitivity to preoperative chemotherapy is a significant prognostic factor for patients with OS (3-5). Chemosensitivity of patients with OS is assessed according to the percentage of tumor necrosis (4,5) or remaining viable tumor cells in resected tumor specimens (3). It is not specific to chemotherapy regimen or drug used. Predicting chemosensitivity at an early stage of diagnosis is a major challenge in OS treatment. Identification of patients whose tumors have the potential to resist current preoperative chemotherapy prior to treatment initiation would avoid ineffective chemotherapy and allow the use of alternative chemotherapy. Numerous studies employing immunohistochemistry or reverse transcription-quantitative (RT-q)PCR have proposed candidate molecules that are associated with outcomes or chemosensitivity, such as p16 (6), RUNX family transcription factor 2 (RUNX2) (7), C-X-C motif chemokine receptor 4 (8), MDR1 (P-glycoprotein) (9)

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Abbreviations: CCP, Comprehensive Cancer Panel; CDDP, cisplatin; CNV, copy number variation; MAP, methotrexate, adriamycin and cisplatin; OAS, overall survival; OS, osteosarcoma

Key words: chemotherapy, CDK4, drug sensitivity, osteosarcoma, overexpression

and COP9 signalosome subunit 3 (COPS3) (10). Expression array analysis has also been employed to define gene profiles for predicting chemosensitivity of patients with OS; various genes, including those encoding twist family bHLH transcription factor 1, programmed cell death 5 (11), desmoplakin, phospholipase A2 group IIA (12), aldo-keto reductase family 1 member C4, glutathione peroxidase 1 and glutathione S-transferase ω1 (13), have been reported as chemosensitivity predictors. However, no overlapping genes were observed between these aforementioned studies. Therefore, the present study aimed to identify potential genomic markers for the prediction of chemosensitivity in patients with OS using a genomic approach on preoperative biopsy samples.

Materials and methods

Patient eligibility and treatment. The Chiba Cancer Center Tissue Bank includes 124 frozen tumor samples obtained from patients with OS that were treated at Chiba Cancer Center (Chiba, Japan) since 1993. Eligible cases were selected for the present study according to the following criteria: i) Diagnosed with conventional, high-grade OS occurring in the extremity, ii) pre-therapeutic biopsy samples were available, iii) age at diagnosis <25 years, and iv) follow-up was monitored for >5 years for survival cases. Finally, 50 tumor samples that satisfied these criteria were selected for study, Chiba OS (ChOS; Table SI). The present study was approved by the institutional review board of the Chiba Cancer Center (approval no. CCC19-14) and conformed to the provisions of the Declaration of Helsinki. Written informed consent was obtained from patients or legally authorized representatives.

Preoperative chemotherapy consisting of a MAP regimen was administered to all patients as described previously (14). If radiological evaluation by CT or MRI conducted during preoperative chemotherapy revealed enlargement of the primary tumor, ifosfamide was added to the MAP regimen. Definitive tumor resection was performed following preoperative chemotherapy, and chemotherapy response was evaluated histologically using resected tumor according to the relative area of necrosis. A 'good response' was defined as a necrotic area >90% of the tumor; 'poor response' was defined as necrotic tumor area <90%, as previously described (4,14).

Tumor specimen and genomic DNA extraction. All tumor specimens were obtained from pre-therapeutic biopsy samples. The tumor content of frozen samples was histologically confirmed as >80% tumor cellularity using paraffin sections (4-μm thick) obtained from the same biopsy. Genomic DNA was extracted using the standard proteinase K digestion method (15) or AllPrep DNA/RNA Mini kit (Qiagen KK). DNA concentration and purity were determined using a NanoDrop 100ND-1000 Spectrometer (Thermo Fisher Scientific, Inc.).

Array comparative genomic hybridization (aCGH) and data analysis. For aCGH analysis, 500 ng tumor or control DNA was fragmented and chemically labeled with Cy3- or Cy5-dye, respectively. Genomic DNAs extracted from normal placenta tissue was used as control DNA for aCGH. High-resolution aCGH was performed using the Agilent Human Genome CGH Microarray kit 4x44K (Agilent Technologies, Inc.), according to the manufacturer's protocols, and analyzed using Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (version 3.1; Agilent Technologies, Inc.). Data extraction from scanned microarray images was performed using Feature Extraction Software (versions 9.5.3.1, 10.7.3.1 and 11.0.1.1; Agilent Technologies, Inc.). Raw data were subsequently transferred to Agilent Genomic Workbench Software Version 6.5 (Agilent Technologies, Inc.) and further analyzed using ADM-2 algorithm with a threshold of 5.5. In order to find copy number variations (CNVs) that discriminated between patients who demonstrated good and poor responses, differential CNV analyses were performed with P-value <0.01 using Genomic Workbench Software. All final retained CNVs were validated using quality control data of the software. All genomic positions were defined according to the University of California Santa Cruz Human (version hg19; Genome Reference Consortium h37; genome.ucsc.edu/cgi-bin/hgGateway?db=hg19). Microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (ncbi.nlm.nih.gov/geo/; accession no. GSE154540).

Targeted exome sequencing. Targeted exome sequencing of 409 cancer-associated genes in tumor and non-tumor samples was conducted using the Ion AmpliSeq™ Comprehensive Cancer Panel (CCP) (Thermo Fisher Scientific, Inc.). For each sample, 40 ng genomic DNA was amplified and used for library preparation using the Ion AmpliSeq™ Library 2.0-96LV and the Ion Barcode Adaptors 1-96 kits (both Thermo Fisher Scientific, Inc.). Then, the library was quantified using the Ion Library Quantification kit (Thermo Fisher Scientific, Inc.) and diluted to a final concentration of 8 pM. Targeted sequencing was performed using an Ion Proton sequencer (Thermo Fisher Scientific, Inc.) with an Ion PI Chip v2 according to the manufacturer's instructions. The sequencing results were analyzed using Torrent Suite v5.0.4 software (Thermo Fisher Scientific, Inc.) to align the barcoded reads with the human reference genome (hg19) and to generate run metrics, including chip loading efficiency, total reads, and quality. Variant Caller v5.0 and Ion Reporter™ 5.2 software (Thermo Fisher Scientific, Inc.) were used for variant detection (allele frequency >5%) and annotation (The Single Nucleotide Polymorphism Database v146; ncbi.nlm.nih.gov/snp/), respectively. Each variant was confirmed visually using Integrated Genomics Viewer or validated using Sanger sequencing performed on an Applied Biosystems 3730 DNA analyzer with Sequencing Analysis Software v6.0 (both Applied Biosystems; Thermo Fisher Scientific, Inc.). The CNVs of the 409 genes were calculated by normalizing the amplicon coverage data from a tumor sample with that of paired non-tumor tissue. Each copy number ratio (tumor/normal) was demonstrated as a log2 ratio (log fold change). Sequence data have been deposited in the Japanese Genotype-phenotype Archive, hosted by the DNA Data Bank of Japan (accession no. JGAS000282).

Semiquantitative RT-PCR and RT-qPCR. Total RNA was prepared from human tissue and OS cell lines using AllPrep DNA/RNA Mini or RNeasy Mini kit (both from Qiagen KK) and reverse-transcribed using random primers and SuperScript II (Thermo Fisher Scientific, Inc.), according to the

manufacturer's protocols. The synthesized cDNA was subjected to PCR-based amplification using rTaq DNA polymerase (Takara Bio, Inc.). Primer sequences are listed in Table SII.

A Veriti PCR system (Thermo Fisher Scientific, Inc.) was used for PCR amplification. The resultant PCR products were separated on 2% agarose gel and visualized using a UV transilluminator (ATTO Corporation) after ethidium bromide staining. RT-qPCR analysis was conducted using an ABI Prism 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). TaqMan primers and probes for *CDK4* (cat. no. HS00364847_m1) and *β-actin* (cat. no. 4310881E) were purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.). RT-qPCR amplification was performed in a 20 μl reaction volume containing 10 μl 2X TaqMan Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), 1 μl 20X TaqMan primers and probes and 2 μl cDNA. The thermocycling conditions were as follows: Decontamination for 2 min at 50˚C, initial denaturation for 10 min at 95˚C and 50 cycles of PCR for 15 sec at 95˚C and 1 min at 60˚C. The relative standard curve method was used for data quantification (16). All reactions were performed in triplicate.

Immunohistochemistry. All tumor samples were routinely decalcified and fixed with 10% buffered formalin for 12 h at room temperature and embedded in paraffin. Sections (4-μm thick) were deparaffinized and antigens were retrieved by autoclaving in 0.01 M citrate buffer (pH, 6.0) at 97˚C for 60 min. Endogenous peroxidase activity was blocked by exposing the slides to 3% hydrogen peroxide for 15 min at room temperature. The primary antibody utilized was CDK4 (1:500; cat. no. AHZ0202; Invitrogen; Themo Fisher Scientific, Inc.). The slides were incubated for 20 min at room temperature with the primary antibody and subsequently labeled using the EnVision+/HRP system (Dako; Agilent Technologies, Inc.). Immunoreactions were visualized using diaminobenzidine (0.03%, 1 min at room temperature) by light microscope, and the sections were counterstained with hematoxylin (FUJIFILM Wako Pure Chemical Corporation) for 5 min at room temperature.

Cell culture, transfection and drug treatment. Human OS cell lines U2OS and SJSA1 were obtained from the American Type Culture Collection and maintained in DMEM or RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin (all Thermo Fisher Scientific, Inc.) in a humidified atmosphere of 5% CO_2 at 37˚C. For transient expression, *CDK4* cDNA was amplified using PCR from human cDNA libraries incorporating a C-terminal 3X Flag-tag sequence, as described by Tatsumi *et al* (17) and ligated into the *EcoRI* and *XhoI* sites of the pCDNA3 vector (Thermo Fisher Scientific, Inc.). For CDK4 overexpression, OS cells were seeded in 6-well plates (80% confluency) and transfected with 2.5 μg pCDNA3-CDK4 or empty plasmid (control) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature. Following 24 h transfection, DNA damage and apoptosis were introduced by further incubation with 0-60 μM CDDP (Sigma-Aldrich; Merck KGaA) for 24 h in a humidified atmosphere of 5% CO_2 at 37˚C. In order to inhibit CDK4 activity, transfected cells were cultured with 0-15 nM PD 00332991 (CDK4/6 inhibitor palbociclib; Santa Cruz Biotechnology, Inc.) for 24 h in a humidified atmosphere of 5% CO_2 at 37˚C.

Immunoblotting. Whole-cell lysates were separated using SDS-PAGE and transferred to PVDF membranes (Immobilon-P; EMD Millipore), as previously described (17). Membranes were incubated with primary antibodies (all 1:1,000) at room temperature for 2 h and then treated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,500) at room temperature for 1 h. Finally, signals were detected using a LAS-4000 Image Analyzer following incubation with ECL reagent (both GE Healthcare). The following antibodies were purchased from Cell Signaling Technologies, Inc.: Anti-CDK4 (cat. no. #12790), anti-Cyclin D1 (cat. no. #2978), anti-Bcl-2 (cat. no. #2870), anti-caspase-9 (cat. no. #9502), anti-E2F transcription factor 1 (E2F1; cat. no. #3742), anti-retinoblastoma (Rb; cat. no. #9309), anti-phosphorylated (phospho-)Rb (Ser807/811, cat. no. #9308), anti-phospho-Rb (Ser780, cat. no. #9307), anti-phospho-Rb (Ser807/811; cat. no. #9308), HRP-conjugated anti-rabbit (cat. no. #7074) and HRP-conjugated anti-mouse (cat. no. #7076). Anti-Flag (M2) antibody (cat. no. F3040) was purchased from Sigma-Aldrich (Merck KGaA), anti-poly(ADP-ribose) polymerase (PARP)-1 (cat. no. sc-8007) was purchased from Santa Cruz Biotechnology, Inc. and anti-actin (cat. no. 013-24553) was purchased from FUJIFILM Wako Pure Chemical Corporation.

Cell viability and apoptosis detection. Measurement of cell viability was conducted by water-soluble tetrazolium salt (WST)-8 assay (Dojindo Molecular Technologies, Inc.). Apoptosis progression caused by CDDP or palbociclib treatment was monitored by accumulation of cleaved caspase-9 and PARP1 via immunoblotting, as previously described (17).

Statistical analysis. Overall survival (OAS) was calculated using the Kaplan-Meier method, and differences in survival were assessed using the log-rank test and Cox proportional-hazards regression method. OAS was defined as the period from the date of diagnosis to that of death or the last follow-up and described with 95% confidence interval (CI). Comparisons were assessed using χ^2 and Fisher's exact tests for categorical variables and Mann-Whitney tests for continuous variables. RT-qPCR data are presented as the mean (n=3) for triplicate experiments. Cell viability data are presented as the mean ± SD (n=4). All statistical tests were two-sided. Statistical analysis was performed using JMP® version 9.0.2 software (SAS Institute, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Chemosensitivity is a significant prognostic factor for patients with OS. The 5-year OAS for 50 patients with OS was 73% (95% CI, 59-84%). Univariate analysis of clinical factors revealed that histological response to preoperative chemotherapy ('chemotherapy response') and metastasis at diagnosis were statistically significant prognostic factors associated with OAS (P=0.012 and P=0.0058, respectively), whereas chemotherapy regimen

Table I. Univariate and multivariate analysis of prognostic factors for OS.

		Univariate analysis				Multivariate analysis		
Factor	N (%)	5-year OAS, %	P-value	5-year EFS, %	P-value	Risk ratio	95% CI	P-value
Age, years								
≤20	8 (16)	63	0.5400	54	0.6400			
>20	42 (84)	75		50				
Location								
Femur	30 (60)	70	0.4500	56	0.9500			
Other	20 (40)	79		50				
Histological subtype								
Osteoblastic	42 (84)	73	0.6200	55	0.9600			
Other	8 (16)	73		47				
Chemotherapy response								
Good	29 (58)	89	0.0120[a]	65	0.0200[a]			
Poor	21 (42)	52		38				
Metastasis at diagnosis								
Yes	6 (12)	33	0.0058[a]	17	0.0078[a]			
No	44 (88)	79		59				
Chemotherapy regimen								
MAP	25 (50)	88	0.3600	63	0.2000			
MAP-I	25 (50)	60		44				
OAS								
Chemotherapy response, Poor vs. good						3.24	1.31-8.50	0.011[a]
Metastasis at diagnosis, Yes vs. no						4.34	1.35-12.0	0.016[a]
Chemotherapy regimen, MAP-I vs. MAP						1.11	0.44-2.89	0.830
EFS								
Chemotherapy response, Poor vs. good						2.5	1.08-5.96	0.032[a]
Metastasis at diagnosis, Yes vs. no						3.48	1.11-9.21	0.034[a]
Chemotherapy regimen, MAP-I vs. MAP						1.29	0.55-3.17	0.560

[a]P<0.05. OAS, overall survival; EFS, event-free survival; MAP, methotrexate, adriamycin and cisplatin; I, ifosfamide.

(MAP in the presence or absence of ifosfamide) was not associated with OAS (P=0.36) (Table I; Fig. 1). These results were confirmed by multivariate analysis (Table I).

Significant enrichment of copy number gains of 12q14.1 and 19q13.32 in poor responders. Genome-wide aCGH analysis of the pretreatment of OS tissue samples was performed to identify CNVs associated with tumor chemosensitivity. Differential analyses of CNV between good (n=29) and poor responder (n=21) cohorts identified three loci that were significantly associated with chemosensitivity (P<0.01; Table II). Recurrent gain of chromosomes 12q14.1 and 19q13.32 were identified in 5 of 21 (24%) poor responders, whereas these gains were not identified in good responders (0/29, 0%; P=0.0096). A recurrent gain of chromosome 7q36.2-3 was also significantly observed in 8 of 29 (28%) good responders compared with poor responders (0/21; P=0.008). Meanwhile, there was no statistically significant recurrent copy number loss between good and poor responders.

CDK4 is a key gene associated with copy number gain of 12q14.1. It was hypothesized that one or multiple genes in loci with copy number gain, such as 12q14.1 and 19q13.32, contribute to chemo-resistance in poor responders. In order to address this, mRNA levels of 11 genes in gained regions 12q14.1 and 19q13.32 were compared between 24 tumors

Table II. Chromosomal alterations significantly associated with chemosensitivity.

Cytogenetic band	No. of probes	Gain in good responder (n=29)	Gain in poor responder (n=21)	P-value	Genes	Cases
7q36.2-3	13	8	0	0.0080	*DPP6*, *PAXIP1*, *HTR5A*, *INSIG1*	ChOS-5, 25, 27, 31, 39, 63, 76, 82
12q14.1	10	0	5	0.0096	*AGAP2*, *TSPAN31*, *CDK4*, *MARCH9*, *CYP27B1*	ChOS-38, 46, 48, 50, 90
19q13.32	12	0	5	0.0096	*BCL3*, *CBLC*, *BCAM*, *PVRL2*, *TOMM40*, *APOE*	ChOS-21, 34, 48, 50, 83

DPP6, dipeptidyl peptidase-like 6; PAXIP1, PAX-interacting protein 1; HTR5A, 5-hydroxytryptamine receptor 5A; INSIG1, insulin-induced gene 1; AGAP2, ArfGAP with GTPase domain, ankyrin repeat and PH domain 2; TSPAN31, tetraspanin 31; MARCH9, membrane-associated ring-CH-type finger 9; CYP27B1, cytochrome P450 family 27 subfamily B member 1; BCL3, BCL3 transcription co-activator; CBLC, Cbl proto-oncogene C; BCAM, basal cell adhesion molecule; PVRL2, poliovirus receptor-related 2; TOMM40, translocase of outer mitochondrial membrane 40; APOE, apolipoprotein E; ChOS, Chiba osteosarcoma.

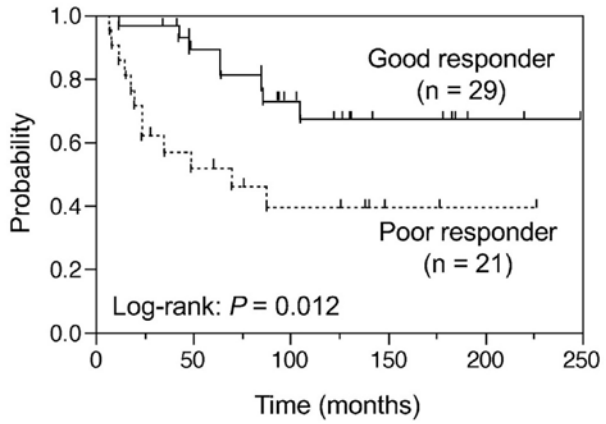

Figure 1. Kaplan-Meier curve for overall survival of patients with OS separated by good or poor response to conventional chemotherapy.

(13 good and 11 poor responders) with available RNA via semiquantitative RT-PCR. *CDK4* in 12q14.1 was the only gene with higher transcript levels that were positively associated with copy number gain (Fig. S1).

In addition, aCGH confirmed that *CDK4* was located in the minimal region of overlap in the gained region. Copy number analysis showed amplification or gain of the *CDK4* locus in poor responders, which was not observed in good responders (Fig. 2A). In order to identify additional markers based on gene mutations, next generation sequencing (NGS)-based targeted exome sequencing of 409 cancer-associated genes was performed with seven and nine tumors from good and poor responder cohorts, respectively. Although 16 and 13 non-synonymous somatic gene mutations were found in the respective cohorts, no recurrent mutation was observed, except for *CDK4* amplification or gain in poor responders (Table SIII). Increased copy number of *CDK4* was also confirmed by calculation of relative read depth of the target exome sequences in three tumors from poor responders (Fig. 2B).

CDK4 transcript levels in primary OS tissue were assessed by RT-qPCR. As expected, higher *CDK4* transcript levels were observed in four patients with 12q14.1 gain compared with those without 12q14.1 gain (Fig. 3A). *CDK4* levels were significantly higher in poor (n=11) than in good responders (n=13; Fig. 3B; P<0.05). Immunohistochemistry analysis was employed to confirm positive nuclear staining of CDK4 in the OS tissue of patients with 12q14.1 gain (Fig. 3C). Thus, *CDK4* was considered a strong candidate genomic prognostic marker and potential therapeutic target in poor responders.

Overexpression of CDK4 in OS cell lines attenuates sensitivity to CDDP. Next, it was investigated whether higher expression of CDK4 in OS cells effectively decreases sensitivity to chemotherapeutic drugs, such as CDDP. Ectopic expression of CDK4 in OS (U2OS and SJSA1) cells was induced via pCDNA3-CDK4 transfection. Following 48 h transfection, increased protein levels of cyclin D1, a binding partner of CDK4, and Bcl-2, a pro-survival factor, were detected in U2OS and SJSA1 cells, accompanied by increased levels of exogenously introduced Flag-tagged CDK4 (Fig. 4A). Transfected cells were cultured in medium containing CDDP for 24 h to induce apoptosis. CDK4 overexpression caused a significant abrogation of the effect of CDDP on OS cell viability compared with control cells (Fig. 4B). Consistent with this observation, apoptotic cell death caused by CDDP was markedly decreased in OS cells overexpressing CDK4, as indicated by immunoblot detection of cleaved caspase-9 accumulation (Fig. 4C).

Overexpression of CDK4 sensitizes U2OS cells to a CDK4/6 inhibitor. Finally, it was investigated whether overexpression of *CDK4* sensitizes OS cells to CDK4/6 inhibitor palbociclib. When Flag-tagged CDK4 was ectopically overexpressed in U2OS cells, there was an increased phosphorylation of Rb at S807/811 and S780 under normal culture conditions (0 nM Palbociclib; Fig. 5A). Subsequent treatment with palbociclib attenuated Rb phosphorylation in a dose-dependent manner. Treatment of CDK4-overexpressing U2OS cells with palbociclib facilitated apoptosis in a dose-dependent manner, as evidenced by cleavage of PARP1 and caspase-9, compared with control cells. Palbociclib also caused a significant decrease in viability of CDK4-overexpressing U2OS cells (Fig. 5B).

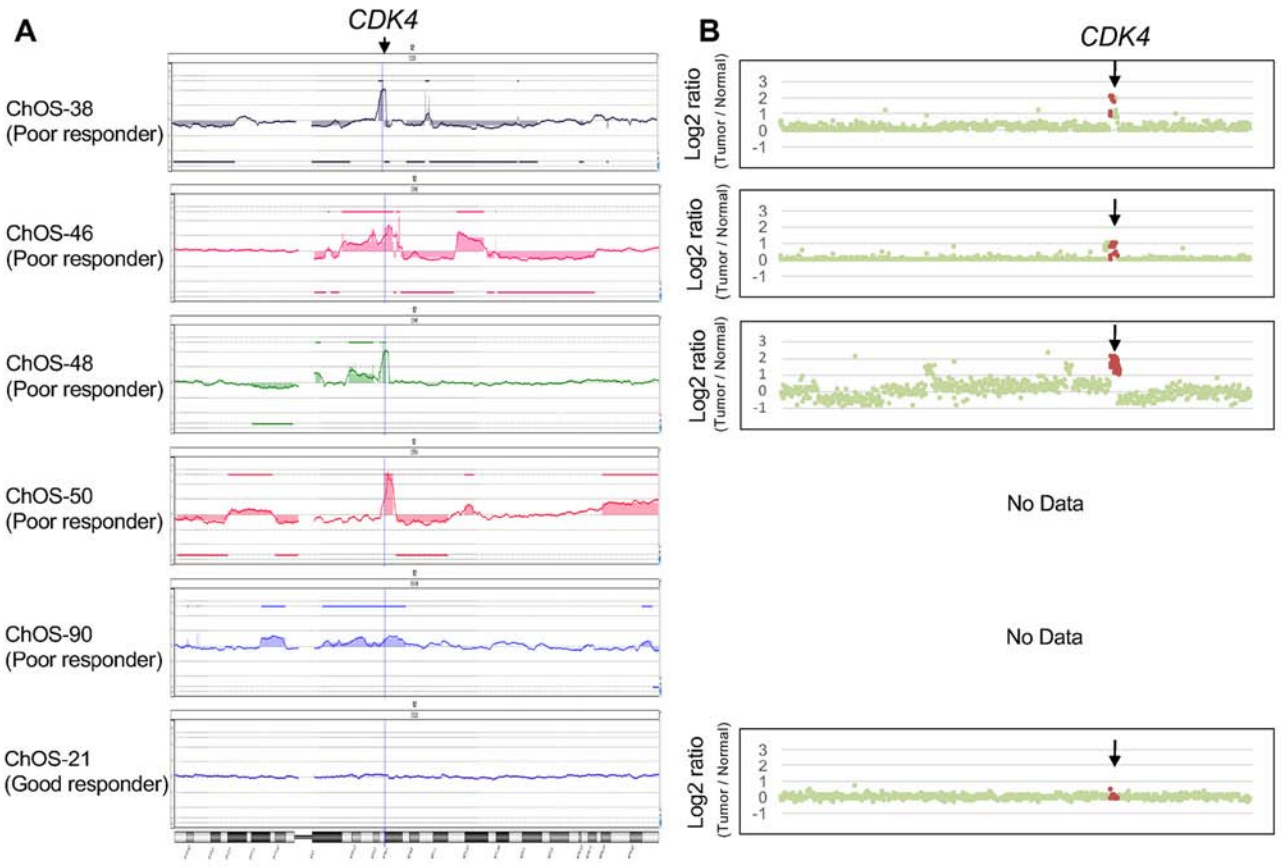

Figure 2. Recurrent copy number gain of the *CDK4* gene in poor responders. (A) Copy number aberrations in chromosome 12 detected by array comparative genomic hybdridization. Representative results of five poor and one good responder are presented. Blue lines indicate positions of *CDK4*. Statistically significant chromosomal regions of loss and gain are indicated by bars below and above copy number data, respectively. (B) Targeted exome sequencing using Comprehensive Cancer Panel confirmed copy number gain of *CDK4* gene in three poor responders. Red dots indicate amplicons corresponding to *CDK4*. ChOS, Chiba osteosarcoma.

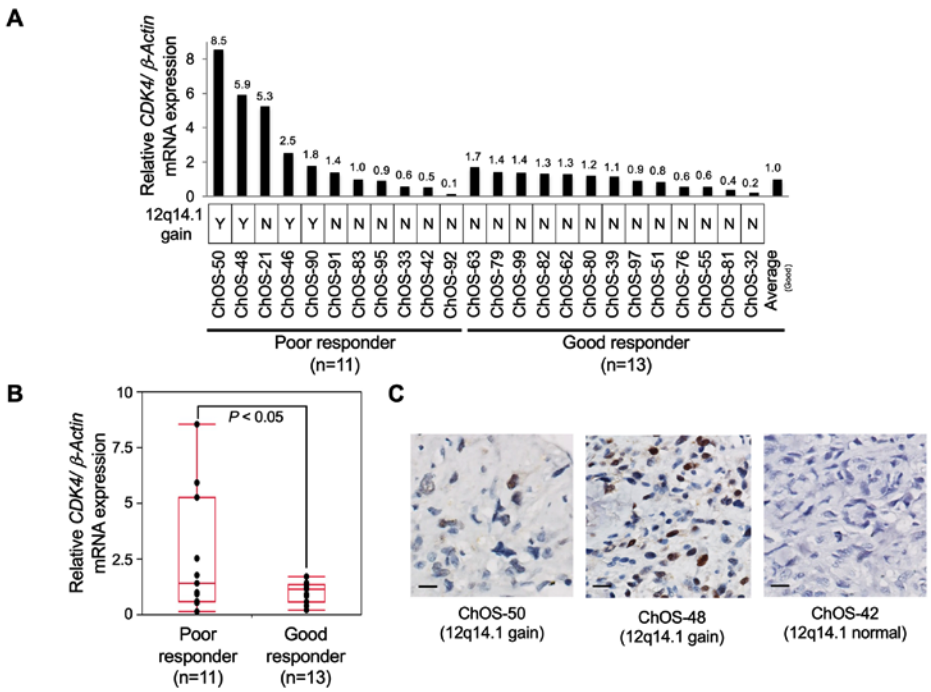

Figure 3. *CDK4* mRNA levels in poor and good responders with 12q14.1 gain. (A) *CDK4* mRNA levels were quantified via reverse transcription-quantitative PCR. *CDK4* expression was normalized to *β-actin*. (B) Boxplots demonstrating *CDK4* mRNA levels in poor and good responders. (C) Immunohistochemical staining for CDK4 in tumor cells (400x magnification). Representative results for high, middle and low expression levels of CDK4 are presented. Scale bar, 50 μm. Y, yes; N, no; ChOS, Chiba osteosarcoma.

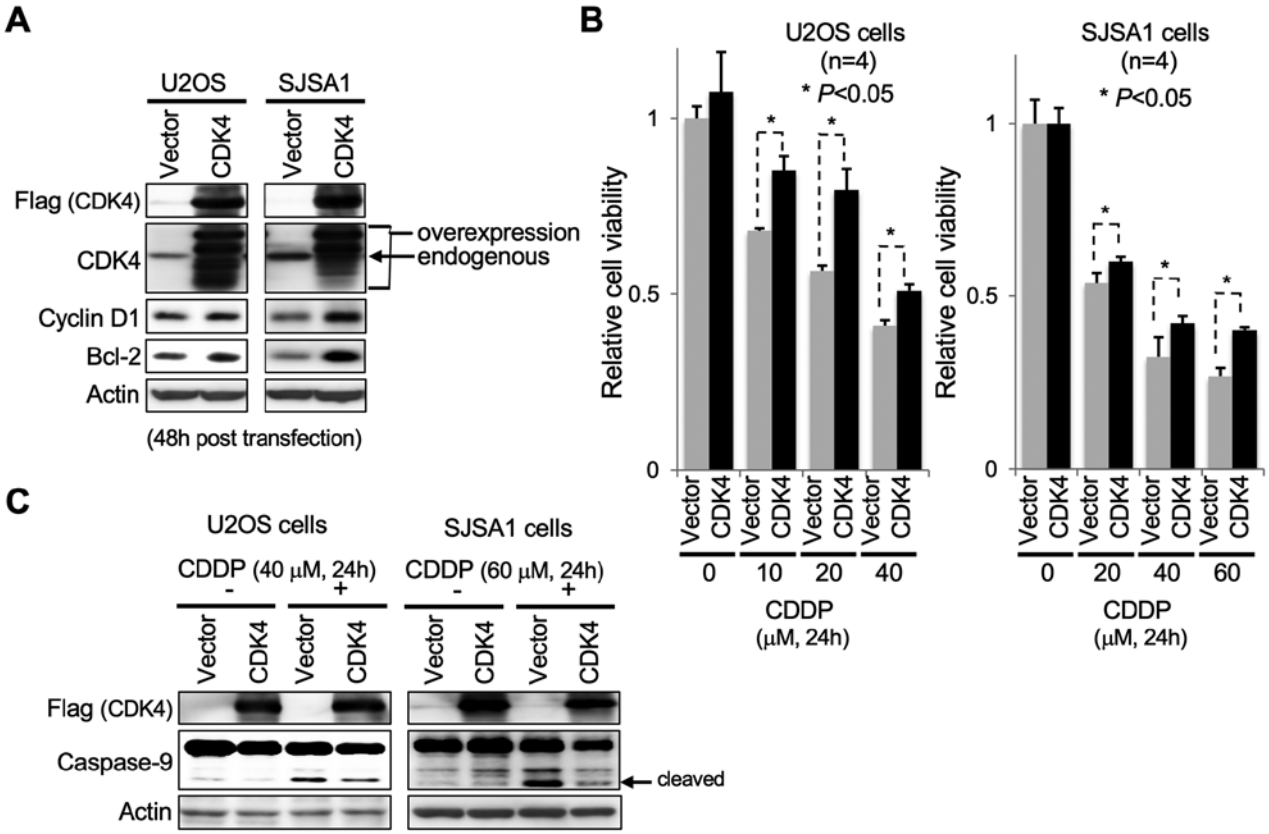

Figure 4. Overexpression of CDK4 in osteosarcoma cell lines decreases sensitivity to CDDP. (A) Immunoblot data showing elevated expression of Bcl-2 and cyclin D1 following ectopic overexpression of CDK4 in U2OS and SJSA1 cells. Actin was used as an internal control. (B) Decreased viability of CDK4-overexpressing cells following treatment with CDDP compared with corresponding vector-transfected controls was assessed by water-soluble tetrazolium salt assay. (C) Cleaved caspase-9 detection via immunoblotting as a substitute for measurement of apoptosis. CDDP, cisplatin

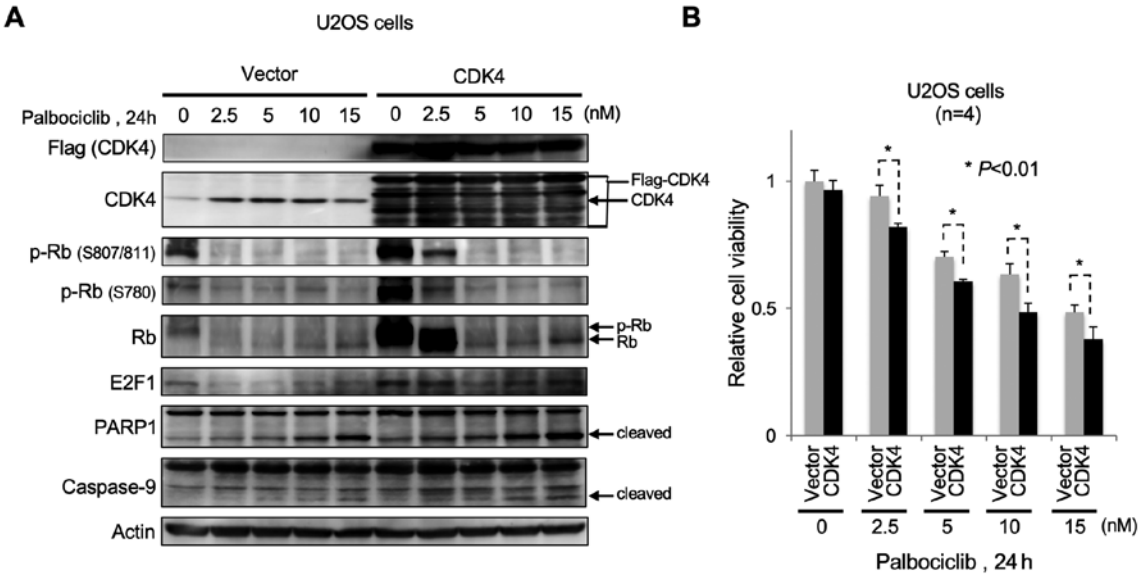

Figure 5. Overexpression of CDK4 in U2OS cells facilitates sensitivity to CDK4/6 inhibitor palbociclib. (A) Immunoblot analysis of CDK4-overexpressing U2OS cells treated with palbociclib. (B) Dose-response of cells treated with palbociclib measuring viability using water-soluble tetrazolium salt assay. p-, phosphorylated; Rb, retinoblastoma; E2F1, E2F transcription factor 1; PARP1, poly(ADP-ribose) polymerase 1.

Discussion

Chemosensitivity to preoperative chemotherapy is a strong prognostic factor for patients with OS (18). The present study investigated genomic markers for the prediction of chemosensitivity in patients with primary OS. Genome-wide CGH array analysis revealed recurrent gain of chromosomes 12q14.1 and 19q13.32 in the poor responder cohort. Targeted exome

sequence and semiquantitative RT-PCR revealed that the *CDK4* gene was associated with 12q14.1 gain and that higher CDK4 transcript levels occurred in tumors with 12q14.1 gain. In functional analysis using OS cell lines, CDK4-overexpressing OS cells exhibited a significant decrease in sensitivity to CDDP and subsequent apoptotic cell death. Conversely, overexpression of CDK4 sensitized OS cells to CDK4/6 inhibitor (palbociclib) and was associated with attenuation of Rb phosphorylation. Based on these results, patients with OS with high expression levels or gain of *CDK4* may be less sensitive to standard chemotherapy containing CDDP and may be good candidates for treatment with CDK4/6 inhibitors.

The present study had two main limitations in the *in vitro* experiments. The first limitation is lack of *CDK4* knockdown experiments in OS cells to confirm the finding that forced overexpression of CDK4 in OS cells attenuated sensitivity to CDDP. Inhibition of CDK4 in OS cells, such as U2OS cells, impedes cell proliferation and promotes G_1 arrest (19), whereas CDDP acts on cells in S phase. Therefore, this could not be assessed using U2OS cells in the present study and should be addressed in future studies using other cell lines, such as Rb- and/or p53-deficient OS cells. The second limitation is that the effects of CDK4 overexpression on proliferation and differentiation of OS cells were not investigated. Here, ectopic overexpression of CDK4 in U2OS cells facilitated Rb phosphorylation. This suggests that CDK4 overexpression promoted proliferation and inhibited differentiation of OS cells via phosphorylation-dependent inactivation of the G_1 regulator Rb. However, significant changes in the viability of OS cells were not detected, regardless of enforced overexpression of CDK4. Future research is necessary to elucidate the precise underlying molecular mechanisms.

Genetic, genomic and NGS analysis have identified molecules and genomic alterations associated with OS development (20,21), although, to the best of our knowledge, few reports have identified predictive markers of chemosensitivity. Cheng *et al* (22) conducted an integrated analysis utilizing publicly available data sets of copy number alterations in pediatric sarcoma cell lines, including OS lines, and identified 33 candidate genes that serve as prognostic markers. They found that genes encoding MDM2 proto-oncogene (MDM2) and GLI family zinc finger 1 (GLI1), which are located in 12q13-15, are predictors of resistance to bleomycin, although this drug is not a standard treatment for patients with OS. Employing whole-exome analysis of samples from eight patients with OS (5 chemotherapy responders and 3 non-responders), Chiappetta *et al* (23) reported mutations in 15 genes that were observed only in non-responders. Although this was a small patient cohort, the findings included genes of interest, such as that encoding Erb-b2 receptor tyrosine kinase 4 (ERBB4), which serves a role in murine double minute X (MDMX)-MDM2 complex stability via MDMX Ser-314 phosphorylation, leading to inactivation of TP53 in poor responders. Zhang *et al* conducted genotyping of ERCC excision repair 1, endonuclease non-catalytic subunit (*ERCC1*) in 260 OS tumors and indicated that *ERCC1* polymorphisms, such as a CC genotype at rs11615 in *ERCC1*, were associated with superior chemosensitivity and better outcomes in patients with OS (24). Although the clinical impact of this polymorphism remains controversial, it may be associated with lower ERCC1 expression, which is also associated with CDDP sensitivity via decreased DNA repair capacity (25). In the present study, NGS identified ERBB3 M1055V and ERCC2 A535V mutations in a tumor with *CDK4* amplification (ChOS-50), but no *ERBB4* mutation. Of the 16 tumors investigated by NGS, three exhibited the *ERCC1* TT rs11615 genotype (potential higher risk), although two were good responders. These candidate markers could not be evaluated in the present study. Since CDDP is currently a key drug for OS, genomic factors, such as DNA damage response genes and *CDK4*, that affect the CDDP sensitivity of OS tumors should be considered as a mechanism.

In the present patient cohort, the amplification of 12q14.1, which harbors *CDK4*, was the most frequent genomic alteration observed only in poor responders. Previous reports have suggested that amplification of 12q13-15, harboring *CDK4* and *MDM2*, may serve an important role in chemoresistance and lead to poor survival in patients with OS (7,26,27). Amplification of 12q13-15 has been observed in 9-25% of all patients with OS (26,28), which is relatively less frequent than aberrations in other loci, such as 17p13.1 (TP53; 70-80%) (29,30), 13q14.2 (*Rb1*; 43-70%) (26,31), 17p11.2 (peripheral myelin protein 22, *COPS3*; 20-78%) (11,32) and 6p21 (*RUNX2*; 24-75%) (11,26,32). Several studies have also reported that amplification of 12q14 is significantly associated with poor event-free survival of OS (26,33). Suehara *et al* (34) reported that pediatric and adult OS tumors comprise at least three tumor subsets exhibiting 4q12 gain with platelet-derived growth factor receptor α (*PDGFRA*) and kinase insert domain receptor amplifications, 6p12 gain with *VEGFA* amplification and 12q13 gain with *MDM2* and *CDK4* amplifications. In the present study, 4q12 and 6p12 gain were observed in 19 (38%) and 18 (36%) tumors, respectively, with no significant enrichment in poor responders. A total of eight cyclin E1 (*CCNE1*) (16%), four *VEGFA* (8%) and four *PDGFRA* amplifications (8%) and 10 CDK inhibitor 2A (*CDKN2A*) homozygous deletions (20%) in 50 OS tumors were detected in the present study. *CCNE1* amplifications and *CDKN2A* homozygous deletions were mutually exclusive in the sample set, as were *VEGFA* and *PDGFRA* amplifications. Again, *CDK4* amplification was only observed in poor responders, whereas the four aforementioned alterations were also observed in good responders. Thus, *CDK4* may be a good marker with high specificity to predict poor responders. For clinical use, further validation with more samples is necessary to determine its suitable cut-off value (relative expression value of 2.5 in RT-qPCR, for example), by comparing the distribution in good responders. A recent report revealed that dedifferentiated OS, which progresses from low-grade OS, exhibits co-expression of MDM2 and CDK4 (35). However, following extensive examination of the whole resected specimen by a bone tumor pathologist, there were no histological low-grade elements in all four cases with *CDK4* amplification in the present study. Dedifferentiated OS has a relatively good prognosis despite poor response to chemotherapy; however, the present four cases with *CDK4* amplification demonstrated poor outcomes, and from the view of clinical behavior, it was hypothesized that these cases might be different from so-called dedifferentiated OS. Concerning 19q13.32 gain, six genes with University of California, Santa Cruz identifiers are located in this region; however, they were not differentially expressed between poor and good responders

in the present study. Unknown microRNAs or long intergenic non-coding RNAs encoded in this region with higher levels in poor responders may exist, the effect of 19q13.32 gain on tumor chemosensitivity remains unknown.

The present study showed that resistance in OS cells was caused by forced overexpression of CDK4. CDK4 forms a complex with Cyclin D1 and promotes G_1 phase progression via phosphorylation-dependent-inactivation of Rb. Previous studies have demonstrated that aberrant activation of Cyclin D1/CDK4 complex promotes anticancer drug resistance in several types of cancer. For example, knockdown of *CDK4* in nasopharyngeal carcinoma cells induces sensitivity to CDDP (36) and overexpression of Cyclin D1 attenuates CDDP sensitivity of testicular germ cell tumor as well as ovarian and prostate cancer (37). CDK4 also promotes resistance to temozolomide (a DNA alkylating drug) in glioma cells (38). Here, OS cells with enforced overexpression of CDK4 significantly increased phosphorylation levels of Rb, indicating that CDK4 overexpression increased cyclin D1/CDK4 activity in OS cells. Thus, it was concluded that CDK4 overexpression promotes CDDP resistance of OS cells, at least in part, by activation of Cyclin D1/CDK4 complex. Further study will uncover the mechanism of how CDK4 abrogates drug sensitivity.

It was hypothesized that CDK4/6 inhibitors exhibit potential efficacy in chemotherapy-resistant OS with 12q14 gain. In hormone receptor (HR)-positive breast cancer, cyclin D overexpression, CDK4/6 activation and subsequent promotion of Rb phosphorylation and E2F release frequently occur, which causes cells to enter S phase and initiate DNA replication (39). Based on this mechanism, CDK4/6 inhibitors have been utilized to treat breast cancer. A Phase III clinical trial demonstrated that CDK4/6 inhibitors exhibit promising antitumor activity and acceptable toxicity for patients with HR^+/human epidermal growth factor receptor 2^- advanced breast cancer (39). Moreover, several clinical trials using various CDK4/6 inhibitors have been conducted for malignant tumors (40), including non-small-cell lung, colorectal and pediatric cancer (41), as well as melanoma, glioblastoma and mantle cell lymphoma (42). Most of the results of these trials were favorable despite varying inclusion criteria, including Rb positivity, cyclin D1 overexpression and *CDK4* or *CDK6* amplification. Also, most of these studies did not require biomarkers as inclusion criteria. Precision medicine based on genomic biomarkers has not been achieved in sarcoma so far. Patients with advanced well-differentiated and dedifferentiated liposarcoma, another malignant mesenchymal tumor that harbors *CDK4* amplification at high frequency (>90%), exhibit favorable outcomes with treatment with a CDK4/6 inhibitor (43). Further studies, including investigation of drug sensitivity and therapeutic effects of a CDK4/6 inhibitor alone or in combination with MAP on CDK4-overexpressing OS cells *in vitro* and *in vivo*, are necessary and assessing CDK4 status in patients with OS may be valuable for guiding precision medicine with a CDK4/6 inhibitor. This may be detected by immunohistochemistry for CDK4. Decalcification, which is required before processing of osteocartilaginous tissue, is known to negatively affect protein quality in formalin-fixed paraffin-embedded (FFPE) samples and subsequent immunohistochemical staining. In order to decrease the negative effect of decalcification in OS samples, a recent alternative method (44), which uses EDTA and short-term formic acid-based decalcification and does not alter antigenicity of the FFPE, would be a better choice for CDK4 immunostaining. In addition, since most cancer gene panel tests for personalized cancer genome medicine contain CDK4 gene loci, *CDK4* information in tumors can be obtained simultaneously. The detection of *CDK4* amplification or overexpression by *in situ* hybridization, RT-qPCR or immunostaining in biopsy or resected specimen may support the therapeutic use of CDK4/6 inhibitors.

The molecular mechanism underlying chemo-resistance in poor responders is still unknown. The present targeted sequencing data identified several candidate genes that may be associated with CDDP chemosensitivity in patients with OS. DICER1 is an essential member of the ribonuclease III family, which controls the maturation of precursor microRNAs, enabling them to function normally (45). In ovarian cancer cells, DICER1 downregulation promotes cell proliferation, migration and cell cycle progression, and Dicer expression is significantly decreased in CDDP-resistant cells (45). Fms-related receptor tyrosine kinase 1 (FLT1; also known as VEGFR1), a member of the VEGFR family, promotes endothelial cell proliferation by activating MAPK or PI3K. Tsuchida *et al* (46) showed that CDDP exposure enhances expression of FLT1 by inducing autocrine signaling, thereby promoting cell survival and expansion of a drug-resistant side population in OS cell lines. Further analysis using meta-data may be necessary to identify other predictive markers and molecular targets for chemoresistance in OS treatment. Poor responders in the present study also exhibited *CCNE1* (n=2), *VEGF* (n=1) and *PDGFRA* amplification (n=2) and *CDKN2A* homozygous deletion (n=6). Although these alterations were not statistically significant markers for chemosensitivity, cyclin E-CDK2, VEGF, PDGFR and CDK4/6 inhibition may be potential therapeutic strategies for these tumors.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SI, YT, HN and MO designed the study, performed the experiments and analyzed the results. SI, YT and MO wrote the manuscript. SI, TY, HKa, TT, YH, HKi and TI provided clinical samples, medical data and survival information. AA and MI performed immunohistochemical analysis. SI, YT and MO confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval was obtained from Institutional Ethics Committee of Chiba Cancer Center (Chiba, Japan; approval no. CCC19-14). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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