# Highly frequent allelic loss of chromosome 6q16-23 in osteosarcoma: Involvement of cyclin C in osteosarcoma

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Received June 13, 2006; Accepted August 14, 2006

**Abstract.** The molecular pathogenesis of osteosarcoma is very complicated and associated with chaotic abnormalities on many chromosomal arms. We analyzed 12 cases of osteosarcomas with comparative genomic hybridization (CGH) to identify chromosomal imbalances, and detected highly frequent chromosomal alterations in chromosome 6q, 8p, 10p and 10q. To define the narrow rearranged region on chromosome 6 with higher resolution, loss of heterozygosity (LOH) analysis was performed with 21 microsatellite markers. Out of 31 cases, 23 cases (74%) showed allelic loss at least with one marker on chromosome 6q. We identified two distinct commonly deleted regions on chromosome 6 using markers D6S1565 located at 6q16 and 6q23MS1 at 6q23. The expression analysis of genes located at the deleted region was performed, and the decreased mRNA expression of the *CCNC* gene, one of the regulators of cell cycle, was detected. Growth of osteosarcoma cell line was significantly suppressed after the *CCNC* cDNA transfection. Fine mapping of the deleted region containing a possible tumor suppressor gene and the transfection assay suggest that the *CCNC* is a candidate tumor suppressor gene.

## Introduction

Osteosarcoma is the most common primary malignant tumor of bone, occurring mainly in the extremities of young adolescents. Molecular genetic research in osteosarcomas has revealed various information on the chromosomal alterations, including studies that osteosarcoma have no correlation with any specific translocations or specific chromosomal rearrangements (1,2). It has been reported that both RB1 and TP53 pathways are inactivated, and the regulation of cell cycle is impaired in most osteosarcomas (1). For example, deletion/mutation of the *CDKN2A* gene encoding both $p16^{INK4A}$ and $p14^{ARF}$ on 9p21 (3-5), amplification of the *CDK4* (6), *CCND1*, *MDM2* genes (4), and other aberrations related to inactivation of these pathways have been found. Tumor suppressor genes (TSGs) are suspected to be involved in tumorigenesis of osteosarcoma. Loss of heterozygosity (LOH) studies have detected frequent allelic loss at 3q, 13q, 17p and 18q (7). Inactivation of the *RB1* and the *TP53* genes may reflect LOH at 13q and 17p, respectively, and the reduced expression of the *DCC* gene on 18q was reported (8).

Comparative genomic hybridization (CGH) has been utilized as a method to gain an overview of all unbalanced chromosomal alterations. These studies can reveal a very complex pattern of DNA copy number changes in osteosarcoma (9-11). However, because of the low resolution that CGH provides for chromosomal loss, the loss region is too wide to identify the loci of the target TSGs.

In the present study, we performed CGH in 12 cases of osteosarcomas, and defined the narrow mapping of allelic loss on chromosome 6 in 31 cases of osteosarcomas by LOH analysis, in order to identify the loci of putative TSGs. Furthermore, we examined the expression and mutational analyses of the candidate genes.

## Materials and methods

*Tissue samples and cell lines.* Tumor samples were obtained from the Department of Orthopaedic Surgery, Okayama University Hospital, after the acquisition of written informed consent from all patients concerned. Three osteosarcoma cell lines, Y-OS, F-OS and OQ-OS, were established from primary osteosarcoma at the Department of Orthopaedic Surgery, Okayama University (Okayama, Japan). Osteoblast cell line hFOB1.19 was obtained from American Type Culture Collection (ATCC).

*DNA and RNA extraction.* Genomic DNAs were extracted from tumor specimens and corresponding normal tissues (muscles) from the same patient according to SDS/proteinase

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*Key words:* osteosarcoma, chromosomal aberration, comparative genomic hybridization, loss of heterozygosity, cyclin C

K treatment, phenol-chloroform extraction, and ethanol precipitation. Total RNAs from tumor specimens and cell lines were prepared by using a modified acid guanidinium phenol chloroform method (Isogen; Nippon Gene Co., Tokyo, Japan).

*Comparative genomic hybridization (CGH).* Normal metaphase spreads for CGH were prepared by routine procedures. Genomic DNA from tumor samples was labeled by nick translation method with Supectrum Green-dUTP®, and Spectrum Red® normal female or male total human genomic DNA was used as a gender-matched normal reference DNA (Vysis, IL, USA). Total 600 ng of each labeled DNA probes were used in 10 $\mu$l of hybridization mixture (containing 55% formamide, 2X SSC and 10 $\mu$g human Cot-1 DNA) after denaturing at 75˚C for 5 min. After denaturing of the slide in 70% formamide, 2X SSC at 75˚C for 4 min, hybridization was performed with probes at 37˚C in a moist chamber for 72 h. The slide was washed in 50% formamide, 2X SSC at 45˚C for 2 min, in 2X SSC at room temperature for 2 min, and counterstained with 125 ng/ml DAPI in an anti-fade solution (Vysis, IL, USA). The hybridized metaphase chromosomes were analyzed using Leica Cytogenetic workstation® (Leica, Japan). Three images of each metaphase were captured using filter wheel-mounted, single band excitation Rhodamine, FITC, and DAPI filters and analyzed using CGH analysis software (Cyto Vision™ version 4.1, Applied Imaging, UK). Ten metaphases were analyzed to generate fluorescence ratio profiles for each case. The profiles were interpreted according to program guidelines. The thresholds used for interpretation of gains and losses for DNA sequence copy number were defined as a tumor/reference ratio >1.25 and <0.75, respectively.

*Loss of heterozygosity (LOH) analysis.* Eighteen microsatellite markers on chromosome 6 are available through the internet genome database (http://gdbwww.gdb.org/) for LOH study. The other microsatellite markers were analyzed with sense primer: 5'-CGT TTT CAT ACT CTA AGG AGC AA and antisense primer: 5'-TTA AGG TGA TGC CTT TAA AGA GG for 6q16MS2, sense primer: 5'-AGA TAG GGA TGT GGA AAC TGT and antisense primer: 5'-TAT CCT ACC CCC AGA GCA AAT for 6q21MS1, and sense primer: 5'-GAA ACT AGA GGG GTG GCT TAC and antisense primer: 5'-ACC ACG GTT GAG AAG GGG TG for 6q23MS1. Sense primer was labeled with 5-iodoacatamidefluorescein at the 5' end. PCR was carried out in 20 $\mu$l of reaction mixture with 10 pmole of each primer, 50 ng of genomic DNA, 1X PCR buffer, 200 $\mu$M of each deoxynucleotide triphosphate (dNTP), and 0.5 units of Taq DNA polymerase (Takara, Tokyo, Japan). The PCR products were applied on ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA) and analyzed by GeneScan analysis software version 3.7 (Applied Biosystems). LOH was scored if one heterozygous allele showed at least 50% reduced intensity in tumor DNA compared to the corresponding normal DNA.

*Real-time RT-PCR.* Total RNA was reverse-transcribed using ReverTra Ace kit (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. Each cDNA was amplified in 25 $\mu$l of mixture containing 20 pmole of each primer and SYBR Green PCR reagent (Applied Biosystems) on GeneAmp 5700 real-time PCR (Applied Biosystems). The primers are a forward primer: FW 5'-TGA TTG CTG CTG CTA CTT CTG TA on exon 5 and a reverse primer: RV 5'-CAT TAC TGA AAT CTG TCC AAT GG on exon 12 for the *CCNC* gene, and a forward primer: 5'-ACC ACA GTC CAT GCC ATC AC and a reverse primer: 5'-TCC ACC ACC CTG TTG CTG TA for the glyceraldehyde phosphate dehydrogenase *(GAPDH)* gene. Data were analyzed by GeneAmp 5700 SDS Manager software. The quantity of the genes was divided by that of the *GAPHD* gene as an internal control.

*Mutation analysis.* We examined 12 exons encoding the *CCNC* gene for mutation analysis by direct sequencing. The coding region was amplified by RT-PCR using two overlapping pairs of primers as follows: FW-A 5'-ACG GGC TGG GTC TAT GGT CG and REV-A 5'-GAC CTT TGC TCC AGT ATG TGC, and FW-B 5'-TGA TTG CTG CTG CTA CTT CTG T and REV-B 5'-CCA TTG GAC AGA TTT CAG TAA TG. Both strands of the product were directly sequenced using ABI Prism 3100 DNA sequencer (Applied Biosystems).

*Plasmid construction and transfection.* The coding region of the *CCNC* full-length cDNA was amplified with sense primer: S1 5'-GGA TCC GCT GGG TCT ATG GTC GCT C and antisense primer: AS1 5'-GTC GAC TAT GGA ATT CTT CGG by PCR, cloned into pBluescript, sequenced, and sub-cloned with *Bam*HI and *Xho*I restriction enzymes into pcDNA3 expression vector bearing FLAG-tag. For the transfection, subconfluent cultures of Y-OS osteosarcoma cells were transfected with pcDNA-CCNC or pcDNA empty plasmid using Effectene (Qiagen) according to manufacturer's instructions. The culture medium was changed to the selection medium containing 400 $\mu$g/ml G418 at 48 h after transfection. Cell medium was changed every 4 days. After 2 weeks of selection, individual colonies were isolated and cultured. The expression of the exogenous *CCNC* gene in the individual colonies was analyzed by RT-PCR with the *CCNC* sense primer S1 and vector antisense primer: BGH2 5'-TAG AAG GCA CAG TCG AGG CT (for 634 bp), and by Western blotting with anti-FLAG antibody.

*Western blotting.* Cell were lyzed in lysis buffer [50 mM Tris-HCl (pH 7.5)], 150 mM NaCl, 0.5% NP-40, 1 mM PMSF, 1 $\mu$g/ml pepstatin A, 2 $\mu$g/ml aprotinin, 2 $\mu$g/ml leupeptin), and centrifuged at 6,000 rpm for 3 min. The supernatant was quantified by Bradford assay, proteins (50 $\mu$g) were run on SDS-PAGE and transferred onto PVDF membrane (Clear blot membrane-P; ATTO Inc., Tokyo, Japan). The membrane was blocked with 1X PBS containing 5% BSA, and reacted with anti-FLAG antibody (M2; Sigma). Proteins were detected by Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA) and CDP-star on Luminoimage analyzer LAS1000plus (Fujifilm, Tokyo, Japan).

*Cell growth assay.* The transfected cells with pcDNA3-*CCNC* were confirmed by RT-PCR and Western blotting for the expression of exogenous *CCNC* gene. F-OS transformants with pcDNA3-CCNC and pcDNA3 empty vector were plated as $1x10^4$ cells per a well of 96-well plate and cultured for 24 h,

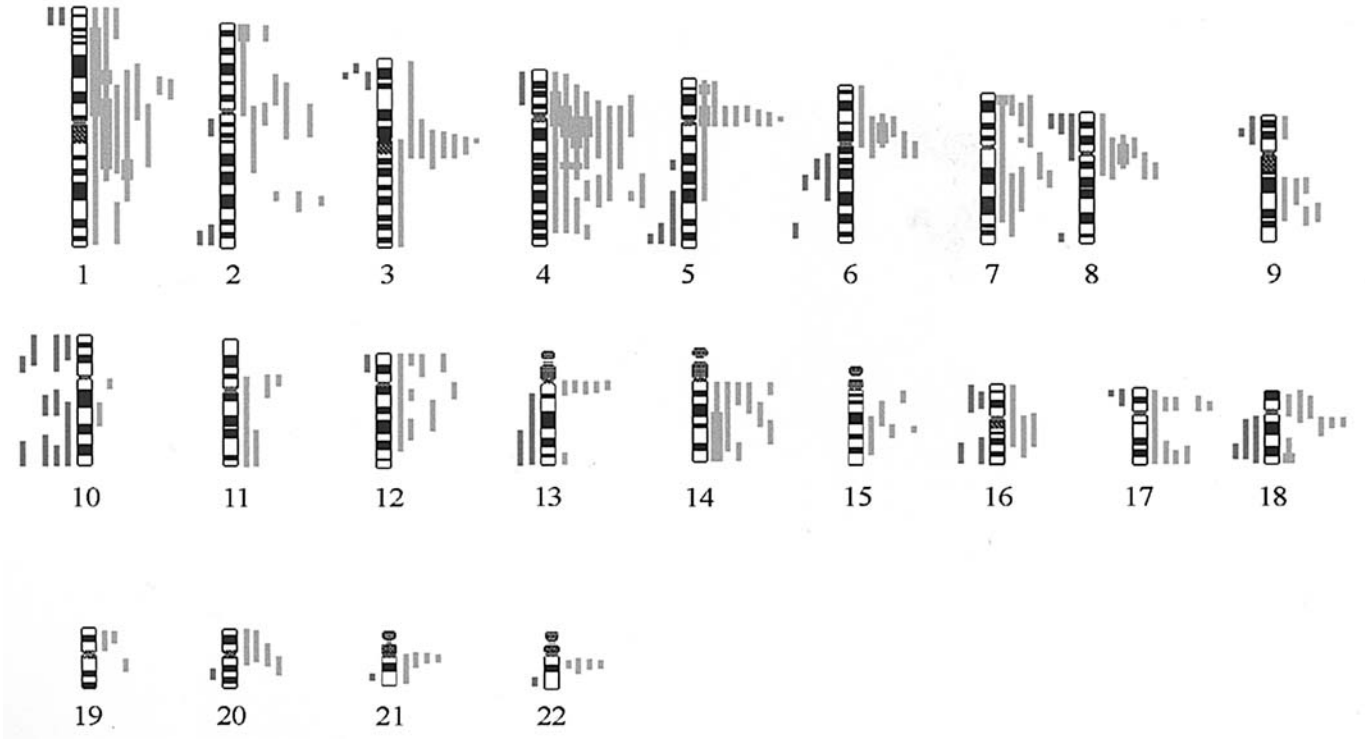


Figure 1. Chromosomal aberrations of 12 osteosarcomas by comparative genomic hybridization. Lines on the left of chromosome ideograms indicate loss, and lines on the right of chromosome ideograms indicate gain. The broad lines are high-level gains.

and then the cell number was counted as day 0. Each of the test samples was assayed in three wells. Cell growth analysis was examined by two group paired t-test. Values of $p<0.05$ were considered significant.

## Results

We analyzed 12 cases of osteosarcomas with comparative genomic hybridization (CGH) to identify chromosomal imbalances. The results are shown on the chromosome ideograms (Fig. 1). Genomic imbalances were detected in all cases. Chromosomal gains were frequently detected on 1p (7/12, 58%), 4p (10/12, 83%), 4q (10/12, 83%), 5q (7/12, 58%) and 8q (7/12, 58%) (Table I). Chromosomal regions involved in chromosomal loss were 10p12-13 (4/12, 33%), 10q 25-26 (4/12, 33%), 6q21 (3/12, 25%), 8p21 (3/12, 25%), 10p14-15 (3/12, 25%), 10q21 (3/12, 25%), and 18q23 (3/12, 25%). Partial loss of chromosome 6q was shown in 4 cases of osteosarcomas, but no gain of chromosome 6q was found in CGH analysis.

To confirm the results with higher resolution, LOH analysis was performed on 31 cases of osteosarcomas using 21 microsatellite markers spanning chromosome 6q15-24. The findings for each of the 21 loci on chromosome 6 are summarized in Fig. 2. Out of 31 cases, 23 cases (74%) displayed allelic loss at least with one marker on chromosome 6q. Among these 23 cases, 3 cases (case no. 15, 25 and 31) showed LOH at all informative loci, suggesting a chromosome loss within a wide range of chromosome 6q. We identified two distinct commonly deleted regions on chromosome 6 with markers D6S1565 located at 6q16 (62%) and 6q23MS1 located at 6q23 (61%).

Table I. Frequent aberrations in 12 osteosarcomas detected by CGH.

| Minimal common regions | Frequency |
|---|---|
| Losses | |
| 10p12-p13, 10q25-q26 | 4/12 |
| 6q21, 8p21, 10p14-p15, 10q21, 18q23 | 3/12 |
| 1q43-q44, 5q34, 6q15-q16, 6q22, 8p23-p22, 9p21 10q22-q24, 13q22-q34, 16p12-p13.1, 16q23-q24 17p13, 18q12-q22, 22q13 | 2/12 |
| Gains | |
| 4p12-p13, 4q12-q13 | 10/12 |
| 4q22-q23 | 9/12 |
| 1p13-p22, 4p14, 5q11.2, 8q12-q13, 8q22-q23 | 7/12 |
| 4q24-q26, 8q11, 8q21.3, 8q24.1, -q24.3 | 6/12 |
| High level amplifications | |
| 4q13 | 4/12 |
| 1p13-p31, 1q22-q24, 4q12, 4q24, 8q21, 2-q24.1, 14q32 | 2/12 |

These results suggest a possibility that tumor suppressor genes located on these regions are inactivated during the development/progression of osteosarcoma. We searched the

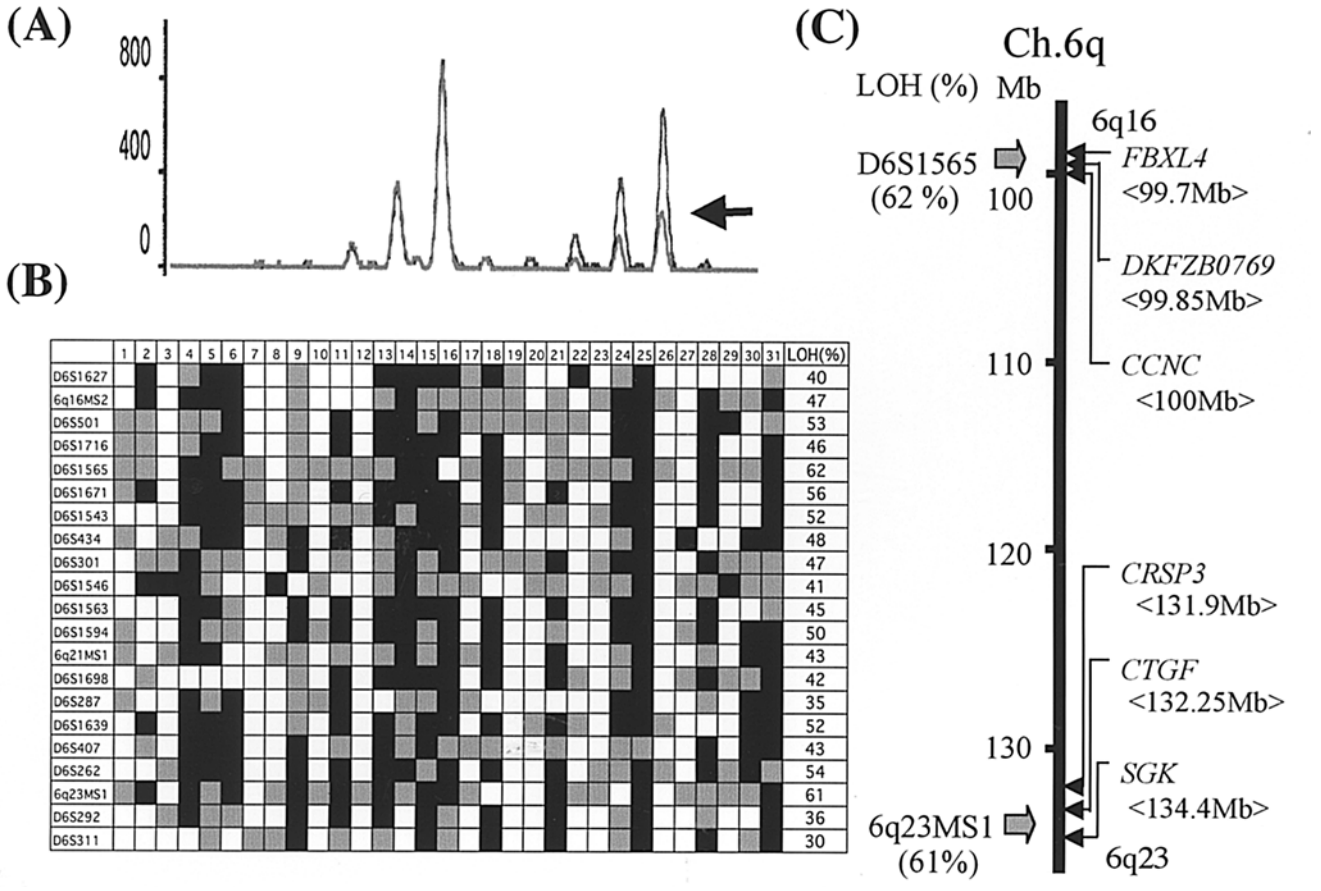


Figure 2. Loss of heterozygosity (LOH) analysis of osteosarcomas. (A) Representative data of LOH. Arrow indicates a reduction of one allele in the tumor DNA as compared with the matched normal DNA. (B) Schematic representation of LOH distribution. Case numbers are shown on top. Microsatellite markers used are shown to the left. Black box, LOH; white box, retention; shaded box, not informative. (C) Candidate tumor suppressor genes around the markers, D6S1565 and 6q23MS1, showing frequent LOH. Approximate nucleotide number from the short arm telomere is shown. Mb, megabase.

genome database on National Human Genome Research Institute (http://www.genome.gov/) and defined the map of this region including the microsatellite markers we used. We found that the candidate tumor suppressor genes *FBXL4, DKFZB0769* and *CCNC* are located around the marker D6S1565, and that the *CRSP3, CTGF* and *SGK* genes are located around the marker 6q23MS1 (Fig. 2C).

By real-time RT-PCR, we quantitatively analyzed the expression level of mRNAs of these genes in 8 osteosarcoma tissue samples, 6 osteosarcoma cell lines and an osteoblast cell line as control. We used the *GAPDH* gene as an internal control. The data are presented as the ratio of these gene transcripts against the *GAPDH* transcript (Fig. 3). The CCNC gene expression was lower in osteosarcoma cell lines as compared with that of osteoblast cell line. The tendency of the decreased expression of the *CCNC* gene was observed in osteosarcoma tissue samples. As mRNA level of the other genes was almost the same or higher than that of osteoblast cell line, we focused further analyses on the *CCNC* gene. Therefore, we examined the mutation of the *CCNC* gene by direct sequencing; however, no mutations was detected in 11 osteosarcoma tissues and 5 osteosarcoma cell lines except for silent mutations (data not shown).

We constructed a *CCNC* cDNA-expression plasmid, and transfected the plasmid and the empty vector into osteosarcoma cell line F-OS that showed the decreased expression of the *CCNC* gene. After G418-selection for two weeks, some single colonies were picked up, cultured independently and confirmed the exogenous expression from the *CCNC* plasmid by RT-PCR and Western blotting (Fig. 4A and B). When the cell growth was examined between the *CCNC*- and vector-transformants, the growth of F-OS cells expressing the *CCNC* gene was significantly inhibited as compared with the F-OS cells transfected with empty-vector (Fig. 4).

## Discussion

We performed a genome-wide analysis of chromosomal alteration in osteosarcomas using CGH analysis, and detected high-frequently genetic alterations in all of 12 cases. Target genes on the chromosome 6q, where chromosomal loss was observed in 4 of 12 cases, remain unknown, while some losses we detected may reflect the inactivation of TSGs already reported. Frequent loss of chromosome 6q have been observed in osteosarcoma (10). Bridge *et al* reported partial or complete loss of chromosome 6q in all 73 cases by cytogenetic studies (2). Furthermore, chromosomal deletions at 6q were found in a variety of neoplasms including gastric carcinoma (12), prostate carcinoma (13,14), and acute lymphoblastic leukemia (15). These findings suggest that alteration of putative TSGs

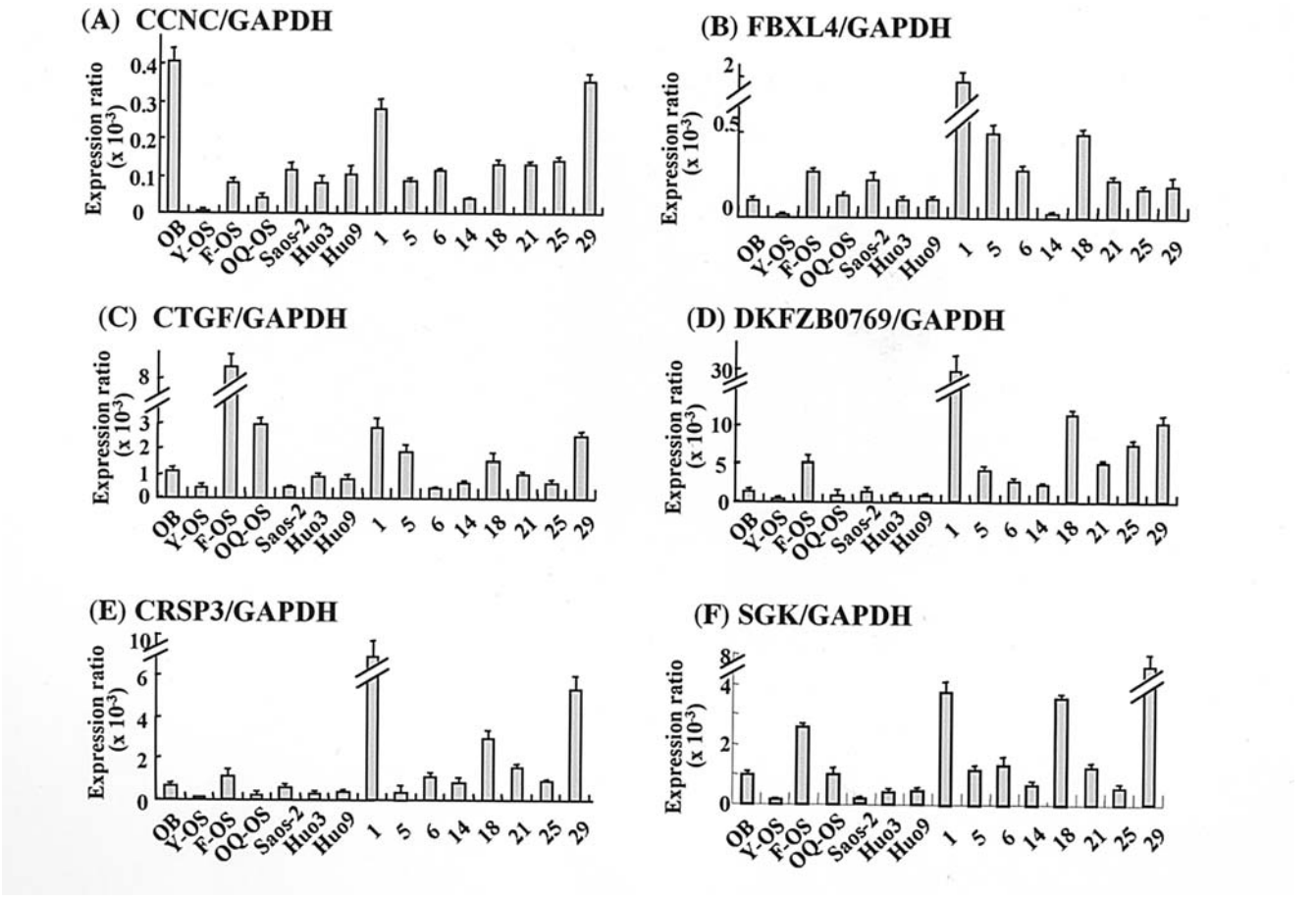


Figure 3. Real-time RT-PCR analysis of candidate tumor suppressor genes in osteosarcomas. The transcripts of the *CCNC* (A), *FBXL4* (B), *CTGF* (C), *DKFZB0769* (D), *CRSP3* (E) and *SGK* (F) genes in 8 osteosarcoma tumor specimens (sample number 1, 5, 6, 14, 18, 21, 25 and 29), 6 tumor cell lines (Y-OS, F-OS, OQ-OS, Saos-2, Huo3 and Huo9) and one osteoblast cell line (OB) were examined by real-time PCR. The data are presented as the ratio of these gene transcripts against the GAPDH transcript.

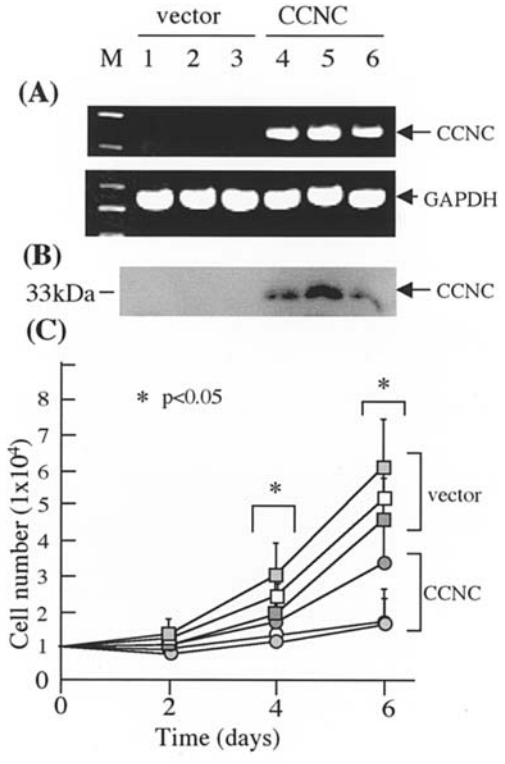


Figure 4. Effect of exogenous *CCNC* gene expression on cell growth of osteosarcoma cells. (A) Detection of the *CCNC* expression in independent colonies of osteosarcoma Y-OS cell transformants with the *CCNC* expression plasmid. The exogenous *CCNC* expression of the transformants was examined by RT-PCR with the *CCNC*-sense primer and vector-antisense primer. Lanes 1-3, empty-vector transformants; lanes 4-6, the *CCNC* transformants; M, molecular weight marker. Upper panel, *CCNC* PCR product (634 bp); lower panel, GAPDH PCR product (450 bp). (B) Western blotting of the osteosarcoma transformants as described above. FLAG peptide-tagged CCNC protein was detected with anti-FLAG antibody as a 33 kDa protein, in only the *CCNC* transformants. (C) Cell growth of osteosarcoma cells with the *CCNC* expression. Cell number of osteosarcoma Y-OS cell transformants with the *CCNC* expression-vector (circle) and with empty-vector (box) was measured by cell counting at the indicated day. Error bars indicate the standard deviation obtained from 3 independent wells.

located in this region may contribute to the tumorigenesis of osteosarcoma.

In order to narrow down the target region including putative TSGs, we performed LOH analysis in 31 cases with 21 microsatellite markers. Three cases displayed LOH for all informative loci, suggesting monosomy of chromosome 6 or total loss of chromosome 6 long arm. The three cases were not contained in the samples we analyzed by CGH. LOH at the

D6S1565 locus (6q16) and 6q23MS1 (6q21) was detected at frequency of 62% and 61%, respectively, suggesting that putative TSGs located on the region are inactivated during the development/progression of osteosarcoma. Based on the recent human genome information, we defined the chromosomal map and listed up the *FBXL4, DKFZB0769* and *CCNC* genes located around the marker D6S1565, and the *CRSP3, CTGF* and *SGK* genes located around the marker 6q23MS1 as candidate TSGs. As the *CCNC* gene exhibited the decreased expression as compared with that of osteoblast cell line, the mutation and expression analyses were performed on the *CCNC* gene. When the *CCNC*-expression vector was transfected into the osteosarcoma cell line that showed the decreased expression of the *CCNC* gene, we found that the transformants exhibited growth suppression as compared with the transformants bearing the empty-vector.

The *CCNC* gene encodes cyclin C. Cdk8/cyclin C is reported to induce phosphorylation of cyclin H, resulting in the downregulation of TFIIH activity and in the inhibition of cellular proliferation (16). Cyclin C is significantly upregulated by 1-α, 25-dihydroxyvitamin D (3) which are known to be inhibitors of cellular growth and inducers of apoptosis (17). Our results support the role of cyclin C as a tumor suppressor, while no mutations were detected by DNA sequencing analysis. Li *et al* also reported that there are no mutations on the retained allele in patients with heterozygous deletion of the *CCNC* gene (18). These results suggest that deletion of one allele is sufficient to promote tumorigenesis as haploid-insufficiency like the $p27^{kip1}$ gene (19) and that epigenetic changes such as methylation may have relevance to inactivation of the *CCNC* gene.

In general, cyclins are considered to be positive regulators of the activity of CDKs. However, it is known that cyclins such as cyclin G play roles not only during progression of the cell cycle, but during differentiation and apoptosis (20). Ren and Rollins showed that cyclin C combines with cdk3 to stimulate pRb phosphorylation during the G0/G1 transition (21). Cyclin C/cdk8 and cyclin C/cdk3 might control cellular proliferation in opposite ways. Further study is necessary to shed light on this issue.

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