Int. J. Cancer (Pred. Oncol.): **79,** 245–250 (1998)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

EXPRESSION OF THE SMOOTH MUSCLE CALPONIN GENE IN HUMAN OSTEOSARCOMA AND ITS POSSIBLE ASSOCIATION WITH PROGNOSIS

Hisako YAMAMURA[1,2], Hideki YOSHIKAWA[3], Masaharu TATSUTA[2], Hitoshi AKEDO[4] and Katsuhito TAKAHASHI[1,5,*]

[1]*Department of Medicine, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[2]*Department of Gastrointestinal Oncology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[3]*Department of Orthopedic Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[4]*Department of Tumor Biochemistry, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[5]*Presto, Japan Science and Technology Corporation (JST), Osaka, Japan*

The basic calponin gene is a smooth muscle differentiation-specific gene that encodes an actin-binding protein involved in the regulation of smooth muscle contractility. We studied the expression of the calponin gene in 8 human osteosarcoma cell lines and 17 primary human osteosarcoma tissues by RT-PCR analysis. We also analyzed mRNA expression of smooth muscle-specific genes including SM22α, caldesmon and α-actin, and for neutral and acidic calponin isoforms. The genes were expressed at various levels by osteosarcoma cell lines and tissues of diverse histological subtypes. The basic calponin protein of an expected size was detected in osteosarcoma cell lines by immunoblot analysis and was localized by immunohistochemistry in the cytoplasm of the tumor cells in osteosarcoma tissues. Survival was found to be significantly increased in patients whose tumors exhibited basic calponin expression, compared with those with no expression. Alterations in the expression of other markers examined were not correlated with prognosis. Our results suggest that the basic calponin gene product may be a novel prognostic variable in patients with osteosarcoma. *Int. J. Cancer (Pred. Oncol.)* 79:245–250, 1998.
© 1998 Wiley-Liss, Inc.

Osteosarcoma is the most common malignancy of bones. The tumors are thought to originate from mesenchymal cells and are classified into 3 major histological subtypes, osteoblastic, fibroblastic and chondroblastic. Several factors are known or suspected to be prognostic factors for this tumor type, including age of onset of disease, sex, location and histological subtype of the tumors, extent of disease at the time of diagnosis and occurrence of metastasis (Davis *et al.,* 1994). Loss of function of the *Rb* and *p53* tumor suppressor genes in osteosarcomas has been correlated with an unfavorable clinical prognosis (Cance *et al.,* 1990). It has been also reported that expression of bone morphogenetic proteins (BMPs) in osteosarcoma cells is associated with an undifferentiated phenotype (Yoshikawa *et al.,* 1994). However, these factors have not provided reliable information to predict the malignant potential and treatment response of a given tumor. Study of a novel molecule that is variably expressed among osteosarcoma cells might provide insight into the mechanisms of tumor progression, as well as a method of identifying tumors with more or less aggressive biological and clinical behavior (Cance *et al.,* 1990).

Calponin is an actin-, tropomyosin- and calmodulin-binding protein originally isolated from smooth muscle (Takahashi *et al.,* 1988, Winder and Walsh, 1990). Structural analysis of cDNAs encoding calponin isoforms has revealed the presence of 3 types of genes with a distinct expressional regulation (Takahashi and Nadal-Ginard, 1991; Strasser *et al.,* 1993; Applegate *et al.,* 1994). Each of the 3 calponin genes encodes distinct classes of isoforms categorized into basic (pI 8 to 10), neutral (pI 7 to 8) and acidic (pI 5 to 6) calponins on the basis of their isoelectric points. The basic calponin gene [also called as the calponin-h1 gene (Strasser *et al.,* 1993)] encodes an originally isolated calponin isoform that is predominantly smooth muscle specific (Takahashi and Nadal-Ginard, 1991). The basic calponin gene expression in smooth-muscle cells during embryonic and post-natal development, as well as in atherosclerotic human arteries, has been recognized as one of the earliest markers of differentiated smooth muscle cells (Duband *et al.,* 1993). The neutral and acidic calponins are expressed in both smooth muscle and non-smooth-muscle tissues (Applegate *et al.,* 1994; Masuda *et al.,* 1996). As to the functional properties of basic calponin, it is capable of inhibiting actomyosin ATPase activity and regulating shortening velocity in smooth muscle contraction. The basic calponin isoform from chicken gizzard induces actin polymerization at low ionic strength and inhibits depolymerization of the actin filaments. It also induces formation of the actin filament bundles. Furthermore, basic calponin binds to myosin, desmin intermediate filaments, tubulin and phospholipids.

During the course of analyzing mice deficient for the basic calponin gene, we found that the endogenous murine basic calponin gene was expressed at high levels in less differentiated osteoblasts in the developing bones of wild-type mice and that homologous recombination knockouts of the endogenous basic calponin gene caused hyperplasia of the osteoprogenitor cells (unpublished observations). These observations suggest that osteosarcoma cells, considered as transformed osteoblasts, may have altered expression of the calponin gene.

In this study, we examined human clonal osteosarcoma cell lines and osteosarcoma tissues to determine whether they expressed the calponin genes and whether the presence or absence of the gene products correlated with the clinical prognosis of patients with osteosarcoma.

MATERIAL AND METHODS

Cell lines and patient materials

Human osteosarcoma cell lines HOS (CRL-1543) and MNNG-HOS (CRL-1547) were purchased from the ATCC (Rockville, MD). HuO9 (JCRB0427), HuO-3N1 (JCRB0413) and HuO-NY (JCRB0614) cells were from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Saos-2 (RCB0428) and OST (RCB0454) cells were purchased from Riken Gene Bank (Tsukuba, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Rockville, MD) supplemented with 10% (v/v) FCS (Bioserum, Canterbury, Australia) and 1% penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% (v/v) CO_2.

Grant sponsors: Ministry of Health and Welfare, Japan (Comprehensive 10-Year Strategy for Cancer Control); Ministry of Education, Science and Culture, Japan (Special Project Research on Atherosclerosis).

*Correspondence to: Department of Medicine, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari-ku, Osaka, Osaka 537, Japan. Fax: (81) 6-972-7749.
E-mail: QYJ02406@niftyserve.or.jp

Received 6 November 1997; Revised 24 December 1997

We studied 17 patients with stage IIB primary osteosarcomas (Enneking *et al.,* 1980) who had been treated at Osaka University Medical School and Osaka Medical Center for Cancer and Cardiovascular Diseases and for whom precise clinical information was available. The clinical database includes the size, location, histological subtype and grade of the tumors, the type of treatment, follow-up time and clinical status. All patients underwent wide local resection and received pre- and post-operative chemotherapy as reported elsewhere (Uchida *et al.,* 1997). Tumor samples used in this series of experiments were obtained from biopsy materials at the time of diagnosis.

RNA preparation

Tumor tissues were frozen immediately after surgical removal and stored at −80°C until extraction of RNA. Total RNA was extracted from cultured cells (70–80% confluency) and osteosarcoma tissues using the Isogen RNA extraction kit (Nippon Gene, Toyama, Japan).

Reverse transcription-PCR analysis

Reverse transcription (RT) of 2 μg of total RNA was carried out using the reaction mixture of Ready-To-Go You-Prime First-Strand Beads (Pharmacia, Uppsala, Sweden) in the presence of 0.2 μg of the random hexamer primer. After 60 min of incubation at 37°C, 0.5 μM of each of the forward and reverse primers, 200 μM of each dNTP mixture and 2.5 U of Taq DNA polymerase (Pharmacia) were added to 8 μl of the first-strand reaction mixture, and then the total volume was adjusted to 50 μl with water. The parameters used for the amplification were 30 cycles of denaturation (94°C, 40 sec), annealing (60°C, 30 sec) and polymerization (72°C, 90 sec). Sequences of the selected forward and reverse 5′-to-3′ primers used, and predicted products size were as follows: basic calponin, GAGTGTGCAGACGGAACTTCAGCC (forward), GTCTGTGCCCAACTTGGGGTC (reverse), 671 bp; neutral calponin, CTGCAGAGCGGGGTGGACATTGGC (forward), GCCGGCCTCCTCCTGGTAGTAAGG (reverse), 519 bp; acidic calponin, GGAAGCGAAGTGCGAGAGACC (forward), CTGTGTGGATCTAATAATCAATGC (reverse), 1061 bp; SM22α, CGCGAAGTGCAGTCCAAAATCG (forward), GGGCTGGTTCTTCTTCAATGGGG (reverse), 928 bp; caldesmon, GTCACCAAGTCCTACCAGAAGA (forward), GCTGCTTGATGGGTCGATTTGA (reverse), 744 bp for low m.w. isoform and 1,508 bp for high m.w. isoform; smooth muscle α-actin, CCAGCTATGTGAAGAAGAAGAGG (forward), GTGATCTCCTTCTGCATTCGGT (reverse), 965 bp; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), CCCATCACCATCTTCCAGGA (forward), TTGTCATACCAGGAAATGAGC (reverse), 731 bp.

The primers for the caldesmon gene were designed to amplify cDNAs encoding both low and high m.w. isoforms. The linearity of the PCR products for calponins, SM22α and caldesmon was obtained between 25 and 35 cycles and for α-actin and GAPDH between 20 and 30 cycles. As a negative control, PCR reactions were conducted with each set of primers, but RNA was omitted in the RT reactions. After agarose gel (1%) electrophoresis in the presence of 0.5 μg/ml of ethidium bromide, the PCR products were revealed by UV irradiation and the image captured and quantitated by Eagle Eye II Still Video System (Stratagene, La Jolla, CA). Variations in signal intensities between different agarose gels were corrected by using those of the m.w. markers in every gel analyzed. To assess relative levels of expression, signal intensity was subjectively graded by 2 independent observers in a blind manner, from (+++), (++), (+) and (−), indicating high, intermediate and low levels, and negative expression. Negative results were repeated at least twice.

Antibodies and immunoblot analysis

Monoclonal antibodies (MAbs) against calponin (clone hCP) and smooth muscle α-actin (clone 1A4) were purchased from Sigma (St. Louis, MO). The specificity of the clone hCP MAb to basic calponin isoform was verified by immunoblot analysis with an alkaline phosphatase staining (Bio-Rad, Hercules, CA) as described previously (Masuda *et al.,* 1996). A polyclonal antibody specific for the SM22α protein was generated in rabbits as described previously (Yamamura *et al.,* 1997).

Immunohistochemistry

The specimens obtained at biopsy were fixed in 3.7% formalin/PBS and embedded in paraffin. Sections 4 μm thick were prepared for staining with hematoxylin-eosin, and for immunohistochemical examination. The sections were mounted on poly-L-lysin-coated microslides, deparaffinized in xylene, dehydrated through graded alcohol and immersed in 70% methanol with H_2O_2 to block endogenous peroxidase. Then, antigen retrieval was performed using an 400-W microwave oven (Toshiba ERT 330, Tokyo, Japan) for 5 min (4 times) in a 10 mM citrate buffer (pH 7.0). The sections were incubated with 1% (v/v) goat serum/PBS for 1 hr at room temperature, washed in PBS and incubated with the anti-calponin MAb (clone hCP) in 2% (w/v) BSA/PBS overnight at 4°C. They were then washed 5 times with 0.005% (v/v) Tween 20/PBS, followed by incubation with the biotinylated goat anti-mouse IgG (TAGO, Camarillo, CA) in 2% (w/v) BSA/PBS for 1 hr at room temperature and avidin-biotin-horseradish peroxidase complex (Vector, Burlingame, CA) for 30 min at room temperature. After being washed in 0.005% (v/v) Tween 20/PBS, the final reaction product was visualized with diaminobenzidine (Wako, Osaka, Japan), and the sections were counterstained with hematoxylin. Tissue samples stained with mouse non-immune IgG were used to assess non-specific staining.

Statistical analysis

Statistical analysis of the differences in the age of onset of the disease and follow-up periods between calponin-positive and -negative groups was conducted using an unpaired *t*-test. The Kaplan-Meier method was used to calculate the continuously disease-free survival functions, beginning from the date of initial diagnosis. In the Kaplan-Meier analysis, the log-rank test was used to estimate the differences in survival between the group of patients. Differences were considered statistically significant with $p < 0.05$.

RESULTS

Expression of calponin isoforms in clonal osteosarcoma cell lines

RT-PCR analysis of the human osteosarcoma cell lines revealed that mRNAs for all 3 types of isoforms, (basic, neutral and acidic calponins) were expressed at various levels in these cell lines (Fig. 1*a*). An mRNA transcript of the basic calponin gene was expressed by the Saos-2 cell line, which has extensive deletion of the *p53* gene (Masuda *et al.,* 1987) and also by the OS^{RB}/N-M cell line, which has loss-of-function mutations in the *p53* and *RB* genes (K. Kawai, personal communications). Complete loss of expression of basic calponin mRNA was observed in a chemically transformed osteosarcoma cell line MNNG-HOS, compared with the related cell line HOS. In contrast, mRNAs for neutral and acidic calponins were expressed in both HOS cells and MNNG-HOS cells. As shown in Figure 1*b,* the basic calponin polypeptide of an expected size (36 kDa) was expressed at detectable levels in both HuO9 and HOS cells, but it was not detected in MNNG-HOS cells (data not shown).

Expression of other smooth muscle differentiation markers in clonal osteosarcoma cell lines

The smooth muscle differentiation-specific genes including SM22α, caldesmon and smooth muscle α-actin were also ex-

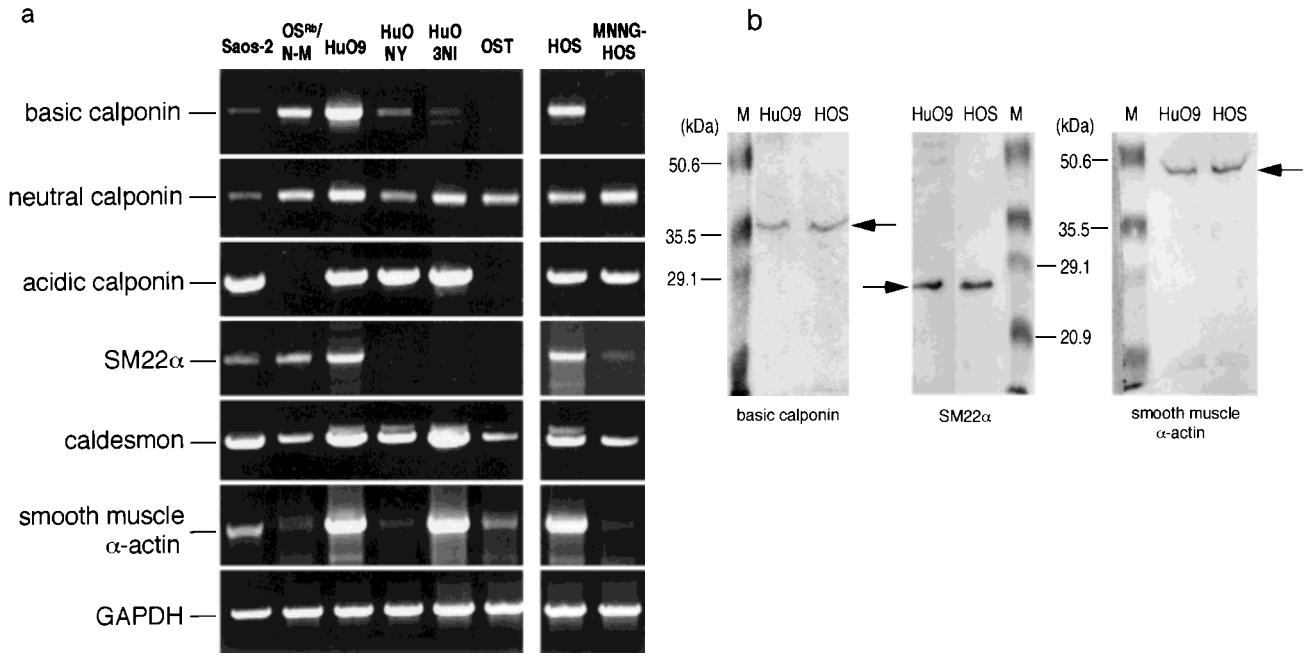

FIGURE 1 – *(a)* Detection of basic, neutral and acidic calponins, SM22α, caldesmon and smooth muscle α-actin expression by RT-PCR analysis in human osteosarcoma cell lines. A major caldesmon PCR product was 744 bp in size, indicating that mRNA expressed in the osteosarcoma cells was a low m.w. isoform. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. *(b)* Detection of basic calponin, SM22α and smooth muscle α-actin gene expression by immunoblot analysis in HuO9 and HOS human osteosarcoma cell lines. Cells cultured on a 10-cm dish (70–80% confluency) in DMEM containing 10% FCS were washed with PBS, dissolved by scraping with a Teflon policeman in 300–400 μl of SDS-PAGE sample loading buffer, containing 8 M urea, 10 μg/ml leupeptin and 0.5 mM phenylmethylsulfonyl fluoride and boiled for 2 min. Samples containing equal amounts of proteins were loaded on 12.5% SDS-polyacrylamide gels. The quantitation of proteins on gels was carried out by Ultroscan XL lazer densitometry (Pharmacia/LKB). Lane M, pre-stained SDS-PAGE standards (Bio-Rad); BSA (50,600), ovalbumin (35,500), carbonic anhydrase (29,100) and soybean trypsin inhibitor (20,900).

TABLE I – SUMMARY OF EXPRESSION OF CALPONINS, SM22α, CALDESMON AND SMOOTH MUSCLE α-ACTIN[1]

Case	Age	Sex	Location	Histology	Metastasis (mo.)	Prognosis (mo.)	Calponin			SM22α	Caldesmon	α-Actin
							Basic	Neutral	Acidic			
1	59	M	Humerus	Osteoblastic	No	Alive (28)	+++	+++	+++	+	++	−
2	14	M	Femur	Osteoblastic	No	Alive (25)	++	+++	++	++	++	+
3	18	M	Fibula	Osteoblastic	No	Alive (47)	++	+	+	+	+	−
4	16	F	Tibia	Chondroblastic	No	Alive (25)	++	+	+	+	++	++
5	18	M	Ilium	Chondroblastic	Yes (28)	Dead (38)	++	+++	++	+++	+++	++
6	36	M	Femur	Osteoblastic	No	Alive (78)	+	++	+++	++	+++	++
7	22	M	Femur	Fibroblastic	No	Alive (14)	+	++	++	++	+++	++
8	24	M	Tibia	Osteoblastic	No	Alive (52)	+	+	+++	++	++	++
9	69	M	Forearm	Fibroblastic	Yes (47)	Dead (54)	+	++	+	+	+	++
10	13	M	Femur	Telangiectatic	No	Alive (8)	+	+++	+	+	+	++
11	15	M	Tibia	Osteoblastic	Yes (25)	Dead (40)	−	+	+	−	−	−
12	13	M	Femur	Chondroblastic	Yes (16)	Dead (30)	−	+	+++	+	+++	+++
13	12	F	Tibia	Osteoblastic	Yes (2)	Dead (14)	−	++	+	++	++	++
14	14	M	Tibia	Osteoblastic	Yes (16)	Dead (23)	−	++	+	+	+++	++
15	16	F	Femur	Osteoblastic	No	Alive (47)	−	+	−	−	+	+
16	12	M	Femur	Osteoblastic	Yes (20)	Dead (27)	−	+	−	+	++	+
17	14	F	Humerus	Osteoblastic	No	Alive (36)	−	++	−	−	−	−

[1], no expression detectable; +, expressed at low levels; ++, expressed at intermediate levels; +++, expressed at high levels.

pressed in these cell lines at various levels (Fig. 1). All the major caldesmon transcripts expressed in these cell lines were represented as a 744-bp product of mRNA for non-smooth-muscle isoform. Similar to the pattern of the basic calponin expression, SM22α and smooth muscle α-actin mRNA transcripts were reduced in the transformed MNNG-HOS cells (Fig. 1*a*). SM22α and smooth muscle α-actin polypeptides were detected in HuO9 and HOS cells by immunoblot analysis (Fig. 1*b*).

Expression of calponin isoforms and other smooth muscle differentiation markers in osteosarcoma tissues

Of the 17 patients with primary osteosarcoma, 10 (59%) had tumors with expression of the basic calponin mRNA transcript at various levels, whereas 7 patients (41%) exhibited no expression (Table I). Not only osteoblastic, but also chondroblastic, fibroblastic and telangiectatic types of tumors showed expression of the basic calponin mRNA. The neutral calponin mRNA transcript,

however, was detected in all tumor samples examined. The acidic calponin was expressed in 14 of 17 tumor samples at various levels. As summarized in Table I, mRNA transcripts for other smooth muscle differentiation markers including SM22α, caldesmon and α-actin were expressed at various levels in 14, 15 and 13 of 17 tumor samples, respectively.

Correlation between immunohistochemical staining of basic calponin and the expression of mRNA transcript in osteosarcoma tissues

Representative cases expressing various levels of the basic calponin mRNA transcripts are shown in Figure 2. Other smooth muscle differentiation markers, including SM22α, caldesmon and smooth muscle α-actin, and neutral and acidic calponins were constitutively expressed in these tumor samples except that smooth muscle α-actin was not detected in case 1. Immunohistochemical analysis of the representative osteosarcoma tissues are shown in Figure 3. In the tumor expressing the basic calponin mRNA transcript at high levels (case 1 in Fig. 2, Table I), immunoreactive forms of calponin were detected in the cytoplasm of most of the tumor cells (Fig. 3). In contrast, in osteosarcoma with low and negative mRNA expression (case 6 in Fig. 2, Table I and case 11 in Table I), weak and negative staining for immunoreactive calponin, respectively, was detected in a subset of the tumor cells.

Survival analysis

To estimate the prognostic significance of the basic calponin expression in the primary osteosarcomas, we divided the patients into 2 groups: a basic calponin-positive group (n = 10) and a negative group (n = 7) (Table I). The 2 groups did not differ in their pre-operative clinical characteristics including Enneking's surgical stage, size (maximum length 10.5 ± 1.1 cm *vs.* 10.3 ± 0.8 cm, mean ± SE, $p = 0.881$), location and histological subtype of the tumors, serum alkaline phosphatase level (400.2 ± 80.3 IU/L *vs.* 430.0 ± 105.1 IU/L, $p = 0.816$), type of treatment and mean follow-up periods of the patients. The patients in the calponin-negative group were significantly younger than those in the calponin-positive group (age of the onset of the disease, 13.7 ± 0.6 *vs.* 28.9 ± 6.3, $p = 0.037$), whereas there was no difference in the age between survivors (n = 10) and non-survivors (n = 7) (23.2 ± 4.5 *vs.* 21.9 ± 7.9, $p = 0.871$). As shown in Figure 4, the continuously disease-free survival curves according to the basic calponin expression indicate that the basic calponin-positive group had a more favorable prognosis than the basic calponin-negative group ($p = 0.020$). We found no significant correlation between prognosis of the patients and expression of the mRNA transcripts for neutral and acidic calponins, SM22α, caldesmon and smooth muscle α-actin.

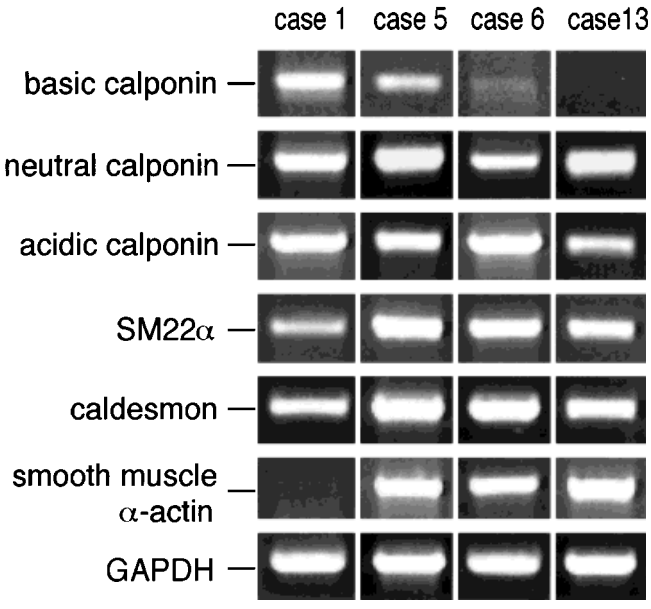

FIGURE 2 – Detection of basic, neutral and acidic calponins, SM22α, caldesmon and smooth muscle α-actin expression by RT-PCR analysis in the representative tumor samples of human osteosarcomas listed in Table I. PCR products were graded according to basic calponin expression from +++ (case 1), ++ (case 5), + (case 6) and − (case 13). The caldesmon PCR product was 744 bp in size, corresponding to a low m.w. isoform.

DISCUSSION

We report here the expression of smooth muscle differentiation-specific genes, calponin, SM22α, caldesmon and α-actin in clonal osteosarcoma cell lines and osteosarcoma tissues of various histological subtypes. Among these smooth muscle differentiation markers examined, we have demonstrated a possible correlation between altered levels of expression of the basic calponin gene in osteosarcoma cells and clinical prognosis of the patients. Survival was found to be increased in a group of patients whose tumor cells had basic calponin expression.

Induction of smooth muscle cell differentiation features in multipotential mesenchymal cells has been identified in the heart and skeletal muscles of the developing embryos, as well as in many pathological settings characterized by tissue remodeling and fibrosis (Sappino *et al.,* 1990). In contrast, in rodent fibroblast strains following transformation to tumorigenicity, the accumulation of smooth muscle α-actin mRNA and α-actin synthesis was greatly reduced (Leavitt *et al.,* 1985). To date, however, relatively little is known about the significance of these altered expression of smooth muscle differentiation features. Osteoblasts are derived from pluripotent mesenchymal cells, which also differentiate into muscle cells, chondrocytes, adipocytes and fibroblasts. It has been reported that BMPs, key environmental signals that induce the osteoblastic phenotype, are capable of inducing ectopic bone formation when implanted into muscular tissues (Urist, 1965). The BMPs convert the differentiation pathway of myoblasts into that of osteoblast-lineage cells (Yamaguchi *et al.,* 1991). BMPs and transforming growth factor-β are capable of promoting differentiation of the neural crest stem cells into mesenchymal cells, which express the smooth muscle differentiation-specific genes calponin and α-actin (Sha *et al.,* 1996). These observations suggest a lineage relationship between osteoprogenitor cells and smooth muscle-like mesenchymal cells. We speculate that transformed osteogenic-lineage cells may be blocked in a proto-differentiated state and may synthesize smooth muscle-specific gene products.

In support of this possibility, our results demonstrate that 7 (88%) of 8 osteosarcoma cell lines (Fig. 1), as well as 15 (88%) of 17 patients with osteosarcomas, were found to be positive for mRNA transcripts of at least 1 of the 3 smooth muscle differentiation-specific genes, basic calponin, SM22α and α-actin (Table I). The same finding was confirmed by the immunohistochemical study of human osteosarcoma tissues. Tumor cells, stained positively for smooth muscle α-actin, were identified in 50% of tumors of various histological subtypes (Hasegawa *et al.,* 1997). It is interesting to note that the patients whose tumors expressed the basic calponin gene were significantly older as a group than those whose tumors did not express the gene (Table I). Since caldesmon mRNA expressed by osteosarcoma cells was invariably a low m.w. isoform (Figs. 1, 2), the pattern of gene expression in osteosarcoma cells resembles that of the dedifferentiated smooth muscle cells. These findings may explain why the expression of osteogenic gene products such as osteopontin, osteonectin and matrix GLA protein has been detected in smooth muscle-derived foam cells in atherosclerotic lesions and in dedifferentiated smooth muscle cells in injured arteries (Giachelli *et al.,* 1993; Ikeda *et al.,* 1993).

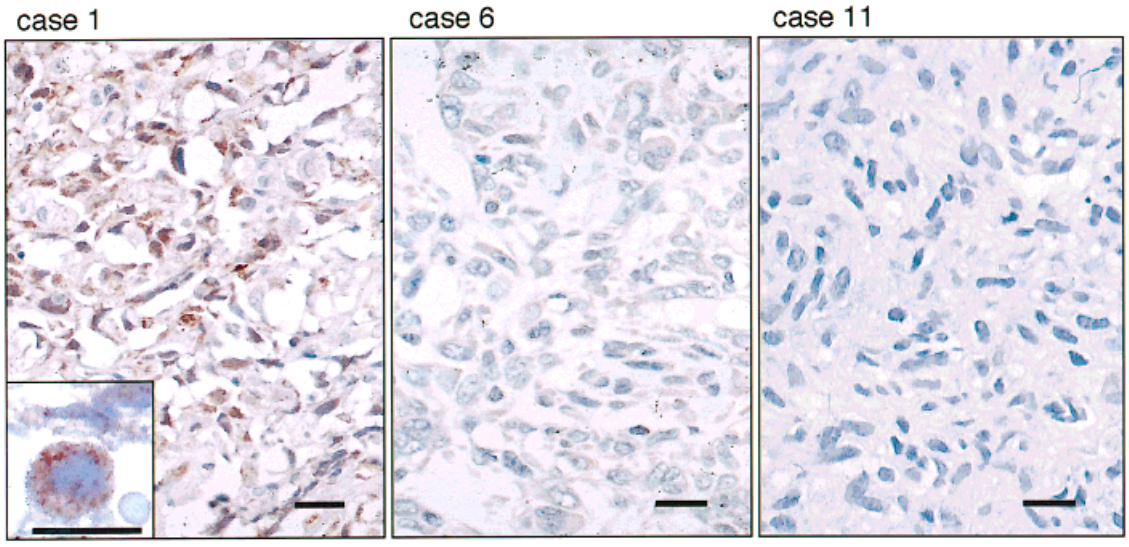

FIGURE 3 – Immunostaining of basic calponin in representative human osteosarcomas categorized in the group of patients with high (case 1), low (case 6) levels and negative (case 11) expression of mRNA (see Table I and Fig. 2). Calponin immunoreactivity was present in the cytoplasm of tumor cells (inset) and correlated with the expression levels of mRNA transcripts. Scale bar, 10 μm.

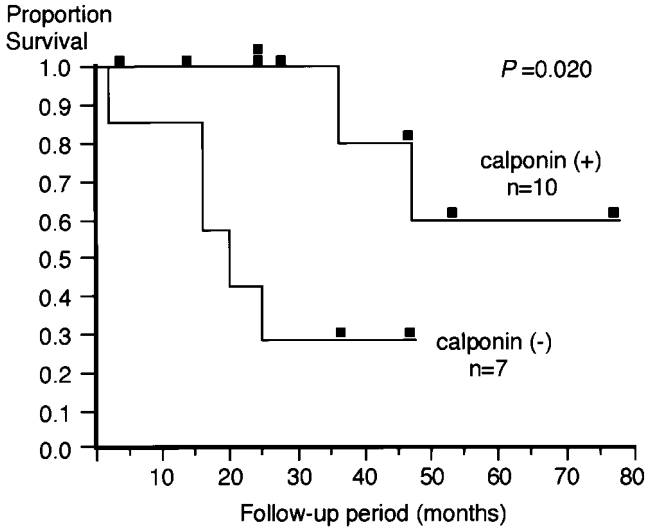

FIGURE 4 – Continuous disease-free survival curve according to basic calponin expression in 17 patients with osteosarcoma. The basic calponin-positive group had a more favorable prognosis than the basic calponin-negative group ($p = 0.020$). Squares indicate survivors.

The finding that metastasis has not been detected in 8 of 10 patients whose tumor cells had basic calponin expression (Table I) implies that the calponin gene product might exert a suppressive effect on tumorigenicity, as well as on the metastatic potential of tumor cells. The basic calponin gene expression was detected in HOS cells but not in MNNG-HOS cells that underwent transformation to tumorigenicity. This is consistent with such a possibility. MNNG-HOS cells are tumorigenic in nude mice showing morphological alterations and an abnormal pattern of growth in culture, while parental HOS cells are not (Rhim *et al.,* 1975). Indeed, we have shown that overexpression of the basic calponin gene in transgenic rats inhibits smooth muscle cell proliferation in balloon-injured arteries (Takahashi *et al.,* 1995).

Of particular interest is the finding that the N-terminal region of calponin shares a characteristic domain structure termed *calponin homology* or CH-domain with the molecules essential for signal transduction of the Ras superfamily of GTPase proteins such as Vav proto-oncogene product and IQGAP1. Removal of the N-terminal 67 residues of the Vav protein, which contains a region of the CH-domain, most strongly conserved compared to the calponin sequence (Masuda *et al.,* 1996), is sufficient to activate the transforming potential of the Vav proto-oncogene products (Coppola *et al.,* 1991; Katzav *et al.,* 1991). The CH-domain in IQGAP1, a potential effector for cdc42Hs/Rac, is required for the co-localization of this protein with actin at the plasma membrane (Hart *et al.,* 1996). The CH-domain is also present in the protein family essential for the regulation of cell shape and the membrane skeleton, including spectrin, cortexillins, BPAGln, α-actinin and dystrophin (Carugo *et al.,* 1997). Furthermore, the agonist-induced activation of isolated smooth muscle cells is accompanied by redistribution of basic calponin protein away from actin filaments in the core of the cell to those in the surface cortex (Parker *et al.,* 1994). Taken together, these observations imply that basic calponin may control cortical actin cytoskeleton and therefore may influence proliferation, the transformed phenotype and the metastatic potential of the tumor cells. It is also possible that loss of the inhibitory action of calponin on actin-myosin interaction may promote the migration and cytokinesis of tumor cells and thus influence its proliferation and metastatic potential.

In conclusion, our data show that smooth muscle genes including basic calponin, SM22α and α-actin are expressed in a subset of human osteosarcomas, indicating a lineage relationship between osteogenic cells and smooth muscle cells. In addition, a favorable clinical outcome of the patients with osteosarcoma has been correlated with basic calponin gene expression in the tumor cells. Although the number of tumors studied is small and the follow-up period is short, our results have implications for the treatment and new subclassification of osteosarcomas.

ACKNOWLEDGEMENTS

We thank Drs. S. Ishiguro and T. Ueda and N. Araki (Osaka) for histological and clinical analyses. We also thank Dr. K. Kawai (Mie) for providing OS^{Rb}/N-M cells.

REFERENCES

APPLEGATE, D., FENG, W., GREEN, R.S. and TAUBMAN, M.B., Cloning and expression of a novel acidic calponin isoform from rat vascular smooth muscle. *J. biol. Chem.,* **269,** 10683–10690 (1994).

CANCE, W.G., BRENNAN, M.F., DUDAS, M.E., HUANG, C.-M. and CORDON-CARDO, C., Altered expression of the retinoblastoma gene product in human sarcomas. *N. Engl. J. Med.,* **323,** 1457–1462 (1990).

CARUGO, K.D., BANUELOS, S. and SARASTE, M., Crystal structure of a calponin homology domain. *Nature (Struct. Biol.),* **4,** 175–179 (1997).

COPPOLA, J., BRYANT, S., TODA, T., CONWAY, D. and BARBACID, M., Mechanism of activation of the *vav* proto-oncogene. *Cell Growth Differ.,* **2,** 95–105 (1991).

DAVIS, A.M., BELL, R.S. and GOODWIN, P.J., Prognostic factors in osteosarcoma: a critical review. *J. clin. Oncol.,* **12,** 423–431 (1994).

DUBAND, J.L., GIMONA, M., SCATENA, M., SARTORE, S. and SMALL, J.V., Calponin and SM22 as differentiation markers of smooth muscle: spatiotemporal distribution during avian embryonic development. *Differentiation,* **55,** 1–11 (1993).

ENNEKING, W.F., SPANIER, S.S. and GOODMAN, M.A., A system for the surgical staging of musculoskeletal sarcoma. *Clin. Orthop.,* **153,** 106–120 (1980).

GIACHELLI, C.M., BAE, N., ALMEIDA, M., DENHARDT, D.T., ALPERS, C.E. and SCHWARTZ, S.M., Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. *J. clin. Invest.,* **92,** 1686–1696 (1993).

HART, M.J., CALLOW, M.G., SOUZA, B. and POLAKIS, P., IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs. *EMBO J.,* **15,** 2997–3005 (1996).

HASEGAWA, T., HIROSE, T., SEKI, K., HIZAWA, K., USUI, M., ISHII, S. and WAKABAYASHI, J., Histological and immunohistochemical diversities, and proliferative activity and grading in osteosarcomas. *Cancer Detect. Prev.,* **21,** 280–287 (1997).

IKEDA, T., SHIRASAWA, T., ESAKI, Y., YOSHIKI, S. and HIROKAWA, K., Osteopontin mRNA is expressed by smooth muscle-derived foam cells in human atherosclerotic lesions of the aorta. *J. clin. Invest.,* **92,** 2814–2820 (1993).

KATZAV, S., CLEVELAND, J.L., HESLOP, H.E. and PULIDO, D., Loss of the amino-teminal helix-loop-helix domain of the *vav* proto-oncogene activates its transforming potential. *Mol. cell. Biol.,* **11,** 1912–1920 (1991).

LEAVITT, J., GUNNING, P., KEDES, L. and JARIWALLA, R., Smooth muscle α-actin is a transformation-sensitive marker for mouse NIH3T3 and Rat-2 cells. *Nature (Lond.),* **316,** 840–842 (1985).

MASUDA, H., MILLER, C., KOEFFLER, H.P., BATTIFORA, H. and CLINE, M.J., Rearrangement of the p53 gene in human osteogenic sarcomas. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7716–7719 (1987).

MASUDA, H., TANAKA, K., TAKAGI, M., OHGAMI, K., SAKAMAKI, T., SHIBATA, N. and TAKAHASHI, K., Molecular cloning and characterization of human non-smooth muscle calponin. *J. Biochem.,* **120,** 415–424 (1996).

PARKER, C.A., TAKAHASHI, K., TAO, T. and MORGAN, K.G., Agonist-induced redistribution of calponin in contractile vascular smooth muscle cells. *Amer. J. Physiol.,* **267,** C1262–C1270 (1994).

RHIM, J.S., PARK, D.K., ARNSTEIN, P., HUEBNER, R.J., WEISBURGER, E.K. and NELSON-REES, W.A., Transformation of human cells in culture by N-methyl-N′-nitro-N-nitrosoguanidine. *Nature (Lond.),* **256,** 751–753 (1975).

SAPPINO, A.P., SCHURCH, W. and GABBIANI, G., Differentiation repertoir of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab. Invest.,* **63,** 144–161 (1990).

SHAH, N.M., GROVES, A.K. and ANDESON, D.J., Alternative neural crest cell fate are instructively promoted by TGFβ superfamily members. *Cell,* **85,** 331–343 (1996).

STRASSER, P., GIMONA, M., MOESSLER, H., HERZOG, M. and SMALL, J.V., Mammalian calponin. Identification and expression of genetic variants. *FEBS Lett.,* **330,** 13–18 (1993).

TAKAHASHI, K., HIWADA, K. and KOKUBU, T., Vascular smooth muscle calponin: a novel troponin T-like protein. *Hypertension,* **11,** 620–626 (1988).

TAKAHASHI, K., MIWA, A., TAKAGI, M., KOJIMA, A., ESHITA, Y., NARA, Y., YAMORI, Y., HIRABAYASHI, M., KATO, O., AWATA, N., KOBAYASHI, T. and SHIBATA, N., Overexpression of calponin in transgenic rats inhibits intimal arterial smooth muscle cell hyperplasia in the carotid artery model of restenosis. *Circulation,* **92,** Abstract (1995).

TAKAHASHI, K. and NADAL-GINARD, B., Molecular cloning and sequence analysis of smooth muscle calponin. *J. biol. Chem.,* **266,** 13284–13288 (1991).

UCHIDA, A., MYOUI, A., ARAKI, N., YOSHIKAWA, H., SHINTO, Y. and UEDA, T., Neoadjuvant chemotherapy for pediatric osteosarcoma patients. *Cancer,* **79,** 411–415 (1997).

URIST, M.R., Bone: formation by autoinduction. *Science,* **150,** 893–899 (1965).

WINDER, S.J. and WALSH, M.P., Smooth muscle calponin: inhibition of actomyosin MgATPase and regulation by phosphorylation. *J. biol. Chem.,* **265,** 10148–10155 (1990).

YAMAGUCHI, A., KATAGIRI, T., IKEDA, T., WOZNEY, J.M., ROSEN, V., WANG, E.A., KAHN, A.J., SUDA, T. and YOSHIKI, S., Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation *in vitro*. *J. Cell Biol.,* **113,** 681–687 (1991).

YAMAMURA, H., MASUDA, H., IKEDA, W., TOKUYAMA, T., TAKAGI, M., SHIBATA, N., TATSUTA, M. and TAKAHASHI, K., Structure and expression of the human SM22α gene, assignment of the gene to chromosome 11, and repression of the promoter activity by cytosine DNA methylation. *J. Biochem.,* **122,** 157–167 (1997).

YOSHIKAWA, H., RETTIG, W.J., TAKAOKA, K., ALDERMAN, E., RUP, B., ROSEN, V., WOZNEY, J.M., LANE, J.M., HUVOS, A.G. and GARIN-CHESA, P., Expression of bone morphogenetic proteins in human osteosarcoma. *Cancer,* **73,** 85–91 (1994).