The Expression of *ccn3(nov)* Gene in Musculoskeletal Tumors

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The CCN3(NOV) protein belongs to the CCN [cysteine-rich CYR61, connective tissue growth factor (CTGF), nephroblastoma overexpressed gene (Nov)] family of growth regulators, sharing a strikingly conserved multimodular organization but exhibiting distinctive functional features. Although previous studies have revealed an expression of CCN3 protein in several normal tissues, including kidney, nervous system, lung, muscle, and cartilage, less is known about its expression in tumors. In this study, we analyzed the expression of CCN3 in musculoskeletal tumors, using a panel of human cell lines and tissue samples. An association between CCN3 expression and tumor differentiation was observed in rhabdomyosarcoma and cartilage tumors, whereas, in Ewing's sarcoma, the expression of this protein seemed to be associated with a higher risk to develop metastases. CCN3 expression was found in 15 of 45 Ewing's sarcoma tissue samples. In particular, we did not observe any expression of CCN3 in the 15 primary tumors that did not develop metastases. In contrast, 15 of the 30 primary tumors that developed lung and/or bone metachronous metastases showed a high expression of the protein ($P < 0.001$, Fisher's test). Our studies indicate that CCN3 is generally expressed in the cells of the musculoskeletal system. This protein may play a role both in normal and pathological conditions. However, the regulation of CCN3 expression varies in the different neoplasms and depends on the type of cells. Thus, as reported for other CCN genes, the biological properties and regulation of expression of CCN3 are dependent on the cellular context and the nature of the cells in which it is produced. Further studies will

help to clarify the biological role of this protein in musculoskeletal neoplasms. (Am J Pathol 2002, 160:849–859)

The CCN3 protein is a member of the emerging CCN [cysteine-rich CYR61, connective tissue growth factor (CTGF), nephroblastoma overexpressed gene (Nov)] family of proteins.¹ Although characterization of the CCN family began a decade ago, these proteins have not received broad attention insofar as interest in them has only recently begun to escalate. This was likely because of the complexity of structure and diversity of functions of the CCN members that have defied simple classification. The CCN family of proteins comprises both positive and negative regulators of growth (CYR61, CTGF, Nov, ELM-1, and COP-1), sharing a common multimodular organization. Family members have been characterized from human, mouse, rat, pig, cow, chicken, quail, and frog and are predicted to have arisen from a common ancestral gene more than 40 million years ago.² The primary translational products of most CCN family members constitute secreted proteins of 35 to 48 kd that contain 38 conserved cysteine residues that are organized into four distinct structural modules: 1) an insulin-like growth factor binding (IGF-BP) motif (GCGCCXXC); 2) a motif represented in the Von Willebrand type C factor, likely to be involved in oligomerization and represented in Von Willebrand factor; 3) a thrombospondin 1 module, represented in thrombospondin and thought to be involved in the interaction with extracellular matrix molecules; and 4) a carboxyl-proximal motif proposed to represent a dimerization domain sufficient to promote interaction of CCN3 with fibulin 1C and CCN2(CTGF).³ Even if the functionality of these domains remains to be

Supported by grants from the Italian Association for Cancer Research, the Italian Ministry for University and Research, the Rizzoli Institute, the Italian Ministry of Health (Ricerca Finalizzata), the Association pour la Recherche sur le Cancer and Ligue Nationale contre le Cancer (Comités du Cher et de l'Indre) (to B. P.); a fellowship from UICC International Cancer Technology Transfer (to S.B.); a fellowship from the Italian Foundation for Cancer Research (to V. C.); a fellowship from the Rizzoli Institute (to R. S.); and a Ph.D. fellowship from the Italian Ministry for University and Research (to A. A.).

Accepted for publication November 14, 2001.

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clearly established, their high conservation might be related to the biological function(s) of these proteins.

The two best-characterized members of the CCN family of proteins, CYR61 and CTGF, are secreted, extracellular matrix-associated proteins that regulate different cellular processes, such as adhesion, migration, mitogenesis, differentiation, and survival. Moreover, they seem to play a key role in angiogenesis, chondrogenesis, and in the development of embryonic skeleton, and have been implicated in wound healing, tumorigenesis, and fibrotic and vascular diseases.^{2,4} The mechanisms through which CYR61 and CTGF might act to execute such a diversity of biological functions are still poorly understood. No specific cell surface receptors for these two proteins have been clearly identified to date, although a receptor-ligand complex of 280-kd molecular size has been reported in a human chondrocytic cell line.⁵ Recent studies have presented evidence that CYR61 and CTGF are ligands of the integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$,^{6,7} suggesting that CYR61 and CTGF may function as adhesive signaling molecules acting through integrin-mediated signaling pathways.^{8,9}

Compared with Cyr61 and CTGF, CCN3 has been studied to a lesser degree and its biological role remains primarily unknown. *ccn3* gene was identified as an aberrantly expressed gene in avian nephroblastoma induced by myeloblastosis-associated virus (MAV).¹⁰ *ccn3* maps on chromosome 8q24.1 and encodes a protein of 48 kd with a secreted signal peptide that is associated with the extracellular matrix.⁴ It shares a homology of ~50% with CYR61 and CTGF. Transcription of the *ccn3* promoter is down-regulated by the expression of the Wilms' tumor suppressor gene WT1¹¹ and the levels of WT-1 and *ccn3* RNAs are inversely correlated in human nephroblastomas,¹² suggesting a role for this protein in kidney development or function.¹³ However, CCN3 was found to be expressed in several other tissues, including nervous system, lung, heart, liver, spleen, thymus, endothelium of blood vessels, and skeletal muscle.^{10,13-16} Moreover, overexpression of CCN3 in cartilage and in mesenchymal tumors is starting to become recognized.¹⁷ In Wilms' tumors, CCN3 is mainly overexpressed in tumors of predominantly stromal origin¹² and is positively correlated with heterotypic muscle differentiation,¹³ further suggesting a role of this protein in the musculoskeletal system. With regard to CCN3 protein functions, no specific biological activity has yet been reported. In chicken embryo fibroblasts, the *ccn3* gene was expressed only in quiescent cells and their entry into the cell cycle, as a result of either the expression of an oncogene (ie, *v-src*) or serum stimulation which led to down-regulation of its transcription.¹⁸ Based on this expression pattern and its growth inhibitory properties,¹⁰ *ccn3* has been proposed to function as a negative regulator of the cell growth. Indeed, in humans, the expression of CCN3 was associated with differentiation of glomerular podocytes during normal nephrogenesis and with tumor-derived mesenchymal differentiation (striated muscle and cartilage) in Wilms' tumors.¹³ However, a recent article indicated that CCN3 could act as a growth factor, stimulating proliferation of 3T3 fibroblast cells and inducing protein tyrosine phos-

phorylation.¹⁴ Therefore, the definition of the role and function(s) of CCN3 in normal and tumoral tissues is still controversial and intriguing.

In this article, we have analyzed the expression of CCN3 in normal mesenchymal cells as well as in several musculoskeletal tumors. Our findings indicate that CCN3 is expressed in cells of the musculoskeletal system and that this protein likely participates in different cellular processes depending on cell type. It seems, in fact, to be associated with differentiation in rhabdomyosarcoma and cartilage tumors and with increased aggressiveness of Ewing's sarcoma.

Materials and Methods

Cell Lines

A panel of 13 osteosarcoma, 12 Ewing's sarcoma, and 5 rhabdomyosarcoma cell lines were analyzed. The osteosarcoma cell lines Saos-2 and U-2 OS, the Ewing's sarcoma cell lines SK-ES-1 and RD-ES, the Askin's tumor cell line SK-N-MC and the alveolar rhabdomyosarcoma cell lines SJ-Rh 30 and SJ-Rh 4 were obtained from the American Type Collection (Rockville, MD). The Ewing's sarcoma cell lines TC-71 and 6647 were kindly provided by T. J. Triche (Children's Hospital, Los Angeles, CA). All of the other osteosarcoma cell lines (SARG, MOS, IOR/OS7, IOR/OS9, IOR/OS10, IOR/OS14, IOR/OS15, IOR/OS17, IOR/OS18, IOR/OS19, and IOR/OS20) as well as the Ewing's sarcoma cell lines here considered (LAP35, IOR/BRZ; IOR/CAR; IOR/NGR; IOR/BER; IOR/RCH; and IOR/CLB) were obtained at the Laboratorio di Ricerca Oncologica, Istituti Ortopedici Rizzoli, Bologna, Italy, and previously characterized.¹⁹ The CCA cell line was obtained from a human embryonal rhabdomyosarcoma.²⁰ The RMZ-RC2 cell line was obtained from an alveolar rhabdomyosarcoma.²¹ The RD/18 cell line is a clone of the commercially available human embryonal rhabdomyosarcoma cell line RD (purchased from Flow Laboratories, VA). The adrenocortical carcinoma NCI-H295R (American Type Collection) were used as positive control for CCN3 detection by Western blotting.¹⁷ Cells were routinely cultured in Iscove's modified Dulbecco's medium supplemented with 20 U/ml penicillin, 100 μ g/ml streptomycin (Sigma, St. Louis, MO), and 10% heat-inactivated fetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel). Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Patients and Tissue Samples

A total of 87 formalin-fixed, paraffin-embedded, musculoskeletal tumor specimens were selected for the study. They included 8 giant cell tumors, 2 chondroblastomas, 9 chondrosarcomas, 13 osteosarcomas, 5 rhabdomyosarcomas, and 45 Ewing's sarcomas. All of the samples were from primary lesions of previously untreated patients. The histology of the primary tumors was reviewed by a pathologist with special expertise in bone tumors (FB). An additional panel of rhabdomyosarcoma cases

Table 1. Clinical Features of the Subgroup of 45 Patients with Ewing's Sarcoma in Whom CCN3 Immunoreactivity Was Assessed and of the Entire Group of Patients with Ewing's Sarcoma

Variable	No. of patients (%)	
	Subgroup (n = 45)	Total group (n = 273*)
Sex		
Male	28 (62)	173 (64)
Female	17 (38)	100 (36)
Age		
≤12 years	14 (31)	96 (35)
>12 years	31 (69)	177 (65)
Tumor site		
Extremity	33 (73)	175 (64)
Pelvis	7 (16)	63 (23)
Other	5 (11)	35 (13)
Local treatment		
XRT	17 (38)	113 (42)
Surgery	17 (38)	105 (38)
Surgery + XRT	11 (24)	55 (20)
Chemotherapy protocol		
REN-1	20 (44)	111 (41)
REN-2	20 (44)	108 (40)
REN-3	5 (11)	54 (19)

*This group includes patients with newly diagnosed Ewing's sarcoma that were seen at the Rizzoli Institute between 1983 and 1993 answering the following criteria of inclusion: a diagnosis of Ewing's sarcoma of bone, age younger than 40 years, absence of metastases at diagnosis, no previous treatment, less than a 4-week interval between the time of biopsy and the beginning of treatment, chemotherapy according to the protocols previously described in detail.²²

Abbreviations: XRT, radiation therapy.

(three embryonal and two alveolar) were accessed from the pathology files of the Hospital for Sick Children. These included both primary sites and metastases to lung and scalp. The Ewing's sarcoma patients were seen at the Istituto Ortopedici Rizzoli between 1983 and 1993 and treated with three consecutive programs of chemotherapy (REN-1, REN-2, and REN-3) that have previously been reported in detail.²² Local treatment consisted of surgery only, surgery followed by radiation therapy, or radiation therapy only, depending on patient age, the site and the size of the tumor, and the necessity to retain the greatest level of function of the tumor-affected size. The present study included 45 patients that were randomly selected for their different clinical course among the larger series of patients seen at the Rizzoli Institute during the same period of time, and for whom tumor samples from the biopsy specimens (obtained before chemotherapy) were available for immunohistochemical analysis. Fifteen patients were disease-free at a median follow-up of 145 months (range, 122 to 192 months), 14 patients had developed metachronous lung metastases at a median time of 19 months after the end of the local treatment, and the other 16 patients had developed metachronous bone metastases at a median time of 18 months after the end of the local treatment. Table 1 summarizes the clinical characteristics of the 45 patients here considered and compares them with the entire group of patients seen at the Rizzoli Institute in the same period of time.

Immunohistochemical Analysis of CCN3 Expression

The immunohistochemistry protocol of Chevalier and colleagues¹³ was used with minor modifications. Five- μ m sections from undecalcified, formalin-fixed, paraffin-embedded tissue samples were placed on poly-L-lysine-coated slides (Sigma). The avidin-biotin-peroxidase procedure was used for immunostaining. Briefly, sections were treated sequentially with xylene and ethanol to remove paraffin. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in methanol for 30 minutes at room temperature. Antigen retrieval by microwave treatment for 15 minutes in an 800 W microwave oven was performed in citrate solution. A blocking step with normal goat serum (Vector, Burlingame, CA) was used. The primary antibody K19M¹³ (diluted 1:400) was applied overnight in a moist chamber at 4°C. The following day, the tissue sections were incubated with secondary biotinylated goat anti-rabbit antibody and with avidin-biotin-peroxidase complex (Vector). The final reaction product was revealed by exposure to 0.03% diaminobenzidine (Sigma), and the nuclei were counterstained with Mayer's hematoxylin. Specimens in which the incubation with the primary antibody had been omitted were used as a negative control. In each experiment, normal kidney tissue was also included as a positive control.

Each case was scored by a pathologist blinded to patient identity. The cases were scored as zero when there was a complete absence of staining for CCN3 protein or when scattered positive cells were observed. The CCN3-positive tumor samples were graded from 1 to 3 according to the distribution of positivity and degree of immunostaining. In particular, score 1 indicated limited positivity (<50% of the specimen) and weak immunostaining; score 2 indicated diffuse positivity (>50% of the specimen) and moderate immunostaining; and score 3 indicated diffuse immunostaining (>75% of the specimen) and strong immunostaining.

Immunofluorescence Analysis of CCN3 Expression

In sarcoma cell lines, CCN3 expression was evaluated by indirect immunofluorescence and cytofluorometric analysis. Briefly, cells from subconfluent cultures were harvested, washed with phosphate-buffered saline (PBS), and fixed with cold methanol for 15 minutes. Cells were incubated with the primary anti-CCN3 polyclonal antibody K19M diluted 1:1200 and, after washing in PBS, with a fluorescein isothiocyanate-conjugated anti-rabbit monoclonal antibody, diluted 1:80 (Technogenetics, Milan, Italy). Cell fluorescence was then evaluated with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) or, for adherent cells, with a QUIPS-XL image analysis system (Vysis Inc., Downers Grove, IL). Differentiation ability of rhabdomyosarcoma cells was studied on cultures maintained in medium supplemented with 2% horse serum. After 5 days, cells were harvested

and analyzed for CCN3 and myosin expression. The percentage of myosin-positive cells was determined after staining with the monoclonal BF-G6 antibody (kindly provided by S. Schiaffino, University of Padova, Padova, Italy), reacting with the embryonic myosin heavy chain.²¹

Western Blotting

Total protein contained in cell-conditioned medium, harvested 72 hours after the cell seeding, was incubated overnight at 4°C with heparin-Sepharose beads 2.5% because the CCN3 protein is glycosylated and binds to heparin. Samples were electrophoresed on a sodium dodecyl sulfate/12% polyacrylamide gel at 130 V for 60 minutes and transferred at 42 V for 90 minutes to nitrocellulose sheets. Blots were incubated with anti-CCN3 polyclonal antibody K19M antibody (1/500 dilution), and then with the horseradish peroxidase-linked secondary antibody (1/5000 dilution; Amersham, Aylesbury, UK), and revealed by enhanced chemiluminescence Western blotting detection reagents (Amersham).

ccn3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was isolated from cell cultures and tissue samples. Extraction of RNA was performed by Trizol extraction kit (Life Technologies, Grand Island, NY). cDNA was obtained from 1 µg of RNA by RT-PCR standard methods using Moloney murine leukemia virus reverse transcriptase (Life Technologies) in the presence of dNTPs and oligo-dT. RT-PCR conditions for human *ccn3* are: forward primer 5'-CACGGCGGTAGAGGGAGATA-3'; reverse primer 5'-GGGTAAGGCCTCCCAGTGAA-3', annealing temperature 60°C. The reaction was performed for 30 cycles. The PCR product is a fragment of 251 bp, separated by electrophoresis on 2% agarose gel. RT-PCR for glyceraldehyde-3-phosphate-dehydrogenase gene (Clontech, Palo Alto, CA) was also performed to demonstrate mRNA integrity. In Ewing's sarcoma cells, EWS fusion transcripts were analyzed by using the primers 22.3, 11.3, and 21.9, previously reported,^{23,24} annealing temperature 60°C.

Statistical Analysis

Fisher's exact test was used for frequency data. Correlations were analyzed using Spearman's test.

Results

Expression of CCN3 in Normal Mesenchymal Cells

A high expression of CCN3 was observed in osteoblasts (Figure 1A), osteoclasts (Figure 1B), chondrocytes (Figure 1C), and muscle skeletal cells (Figure 1D).

Expression of CCN3 in Mesenchymal Cell Lines

A panel of 13 osteosarcoma, 12 Ewing's sarcoma, and 5 rhabdomyosarcoma cell lines was analyzed. Figure 2 shows the expression of *ccn3* mRNA in these cells by RT-PCR. Jurkat cells were used as a negative control.¹⁴ All of the musculoskeletal cell lines expressed *ccn3* mRNA. However, differences were observed in the level of expression, with the osteosarcoma cells showing the highest mRNA levels of expression and the rhabdomyosarcoma cells the lowest. In contrast, the analysis of *ccn3* expression at protein level by indirect immunofluorescence and cytofluorometric analysis gave somewhat different results, confirming that *ccn3* mRNA and protein expression patterns showed an overlap but not a direct correlation.¹³ By cytofluorometric analysis, the highest levels of CCN3 expression were found in rhabdomyosarcoma cell lines (Figure 3). No significant differences were observed between the alveolar and embryonal subtype. Indirect immunofluorescent assays on fixed adherent rhabdomyosarcoma cells indicate the CCN3 protein is widely distributed in the cytoplasm of mononuclear cells, sometimes associated with perinuclear membrane and Golgi apparatus, and highly expressed in fusing myoblasts (Figure 1, E and F). The association between CCN3 expression and myogenic differentiation was demonstrated in the RMZ/RC2 cell line, showing a parallel time-dependent significant increase in the expression of CCN3 and the percentage of myosin-positive cells (data not shown). A variable expression of CCN3 was observed in osteosarcoma cell lines (Figure 4). Five cell lines were completely negative; two cell lines were barely positive whereas a weak but clear positivity was observed in the other six cell lines. The expression of CCN3 in osteosarcoma cell lines was inversely correlated with the expression of alkaline phosphatase, a marker of the osteoblastic differentiation ($r = -0.70$, $P = 0.01$, Spearman's test; data not shown). Among Ewing's sarcoma cell lines, only 3 of 12 showed a weak positivity for CCN3 (Figure 5). Ewing's sarcoma cells carried specific chromosomal translocations.²⁵ The expression of the different chimeric products in the cell lines here considered is reported on Table 2. No significant correlation between expression of CCN3 and the type of hybrid transcripts was observed. However, it is interesting to note that all of the three CCN3-positive cell lines showed the expression of the less frequent types of chimeric products, EWS/FLI-1 type 2 and EWS/ERG.²⁵

To assess the specificity of K19M antibody, Western blot analysis was performed on selected cell lines, representative of CCN3-positive and -negative cells based on data obtained from cytofluorometry (Figure 6A). In parallel, immunohistochemistry was also made on formalin-fixed, paraffin-embedded CCN3-positive or -negative Ewing's sarcoma cells to be sure that this technique may be reliably used in the analysis of tissue samples (Figure 6, B and C).

Expression of CCN3 in Tissue Samples

The expression of *ccn3* mRNA was investigated by RT-PCR in a representative panel of frozen musculoskeletal tumor tissue samples, including 5 giant cell tumors, 1

Figure 1. Immunohistochemical stain for CCN3 in osteoblasts (A), osteoclasts (B), chondrocytes (C), and muscle skeletal fibers (D). E and F show the expression of CCN3 by immunofluorescence in RMZ/RC2 rhabdomyosarcoma cells (E) and in RMZ/RC2-fusing myoblasts, observed after 5 days of culture in differentiated medium (F). Original magnifications, $\times 400$ (A–D).

chondroblastoma, 6 chondrosarcomas, 10 osteosarcomas, 9 Ewing's sarcomas, and 1 rhabdomyosarcoma (Figure 7). The findings obtained were in agreement with what was previously observed in normal mesenchymal cells and musculoskeletal tumor cell lines. In particular, giant cell tumor, a heterogeneous benign neoplasm in which variable numbers of osteoclast-like multinucleated giant-cells are dispersed among mononuclear cells of uncertain histogenesis, showed a generally high expression of *ccn3* mRNA. Chondroblastoma, a benign tumor composed of chondroblasts, and the three cases of chondrosarcoma, a malignant tumor differentiating in cartilage, expressed high levels of *ccn3* mRNA. Accordingly to the data obtained in cell lines by RT-PCR, the expression of *ccn3* mRNA was homogeneously high in osteosarcoma, variable in Ewing's sarcoma, and low in rhabdomyosarcoma.

The expression of CCN3 was also analyzed by immunohistochemistry on paraffin-embedded sections of the different types of musculoskeletal tumors to confirm the data obtained by RT-PCR and to analyze the cellular and tissue distribution of the CCN3 protein. Results are summarized in Table 3.

In giant-cell tumor, an extremely high level of expression was observed in osteoclast-like, multinucleated giant cells, whereas in the mononuclear component of the tumor the immunostaining was weaker (Figure 8A).

In chondroblastoma, the expression was clearly evident in chondroblast-like cells and in the osteoclast-like, multinucleated giant cells that infiltrate the tumor (Figure 8B). Central chondrosarcoma stained positively for CCN3. However, differences in the level of expression were observed among the samples and inside the single tissue samples. In general, the undifferentiated, noncar-

Figure 2. Expression of *ccn3* mRNA in cell lines of rhabdomyosarcoma (A), osteosarcoma (B), and Ewing's sarcoma (C). Jurkat cell line was used as a negative control. Methods for reverse transcription of mRNA and PCR amplification of cDNA fragments were described in Materials and Methods. Amplification products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

tilaginous components showed a weaker immunostaining, whereas the well-differentiated cartilage component expressed CCN3 at extremely high levels (Figure 8, C and D). The two cases of peripheral chondrosarcoma appeared well differentiated and show a very high expression of CCN3.

All of the rhabdomyosarcomas analyzed here overexpressed CCN3 at high levels. We had previously reported that by *in situ* hybridization *ccn3* mRNA seemed comparable to protein levels.²⁶ The expression level of CCN3 seemed to be higher in the alveolar rhabdomyosarcoma than in the subtype of embryonal origin (Figure 8, E and F). In embryonal rhabdomyosarcoma, well-differentiated

Figure 3. Cytofluorometric analysis of CCN3 expression in human rhabdomyosarcoma cell lines. **Open profile** represents cells stained with secondary antibody alone; **filled profile** represents cells stained with the anti-CCN3 antibody. Jurkat cell line was used as a negative control. In each panel, the ordinate represents the number of cells. Data from an experiment representative of at least two similar experiments are shown. Positive cell lines are indicated in **bold**.

cells showed a high expression of the protein, in agreement with previous observations indicating a relationship between CCN3 expression.^{13,26}

With regard to osteosarcoma, all of the primary tumors and lung metastases of patients with osteosarcoma were clearly positive for the protein (Figure 8, G and H).

In contrast with the other mesenchymal tumors, the great majority of Ewing's sarcoma samples were completely negative for CCN3 protein. However, a moderate or even high expression was observed in a few percentages of cases (Figure 8, I and J).

Prognostic Relevance of CCN3 Expression in Ewing's Sarcoma

The prognostic significance of CCN3 expression in Ewing's sarcoma was evaluated by immunohistochemistry on 45 primary tumors that were randomly selected for their different clinical course among a homogeneously treated series of patients referred to the Rizzoli Institute between 1983 and 1993. The patients were followed-up for a minimum of 91 months (median, 144 months). Results were updated in December 2000: 15 patients were free of disease, 16 patients had developed bone metastases, and 14 patients had lung metastases. CCN3 expression was found in 16 of 45 tissue samples (35%). In particular, we did not observe any expression of CCN3 in the 15 primary tumors of patients that did not show clinical progression. In contrast, 16 of the 30 primary tumors that developed lung or bone metastases were positive for CCN3 immunostaining ($P < 0.001$, Fisher's test). In particular, five cases were scored as 1, five as 2, and six as 3 (see Materials and Methods). In the group of tumors that gave metastases, the incidence of CCN3 expression was slightly higher in the primary tumors that developed lung metastases (9 of 14, 64%), compared with tumors that developed bone metastases (7 of 16, 44%). In both of the two subgroups, the incidence of CCN3 positivity was significantly different from that observed in the tumors that did not show evidence of clinical progression (9 of 14 *versus* 0 of 15, $P < 0.001$; 7 of 16 *versus* 0 of 15, $P = 0.007$; Fisher's test).

Discussion

Although sporadic observations have been previously reported on the expression of CCN3 in cells of stromal origin,^{13,17} suggesting that, under normal circumstances, the expression of this protein is associated or required for mesenchymal cells to undergo cartilage, muscle, and neural differentiation, a detailed analysis of its expression in musculoskeletal tumors have not been performed. Results in this study present the first conclusive evidence that CCN3 is generally highly expressed in mesenchymal neoplasms. However, important differences have emerged among the different types of tumors, likely reflecting functional diversities of the protein in different cell types.

Figure 4. Cytofluorometric analysis of CCN3 expression in human osteosarcoma cell lines. **Open profile** represents cells stained with secondary antibody alone; **filled profile** represents cells stained with the anti-CCN3 antibody. Jurkat cell line was used as a negative control. In each panel, the ordinate represents the number of cells. Data from an experiment representative of at least two similar experiments are shown. Positive cell lines are indicated in **bold**.

Expression of CCN3 in the Musculoskeletal System and Derived Tumors

Similar to CYR61 and CTGF,^{27,28} CCN3 was highly expressed in normal human mesenchymal cells of the musculoskeletal system, such as osteoblasts, osteoclasts,

chondrocytes, and skeletal muscle cells, further supporting the idea that the CCN genes might be heretofore underestimated regulators of normal mammalian chondrogenesis and musculoskeletal development. In pathological conditions, expression of CCN3 was widely observed in musculoskeletal tumors, with the only notably

Figure 5. Cytofluorometric analysis of CCN3 expression in human Ewing's sarcoma cell lines. **Open profile** represents cells stained with secondary antibody alone; **filled profile** represents cells stained with the anti-CCN3 antibody. Jurkat cell line was used as a negative control. In each panel, the ordinate represents the number of cells. Data from an experiment representative of at least two similar experiments are shown. Positive cell lines are indicated in **bold**.

Table 2. Expression of EWS Fusion Transcripts and CCN in Ewing's Sarcoma Cell Lines

Cell line	EWS fusion transcripts	NOVH protein (% positive cells)
TC-71	EWS/FLI-1 TYPE 1	1.5
SK-N-MC	EWS/FLI-1 TYPE 1	11.4
IOR/LAP-35	EWS/FLI-1 TYPE 2	31.4
SK-ES-1	EWS/FLI-1 TYPE 2	3.3
6647	EWS/FLI-1 TYPE 2	85.5
IOR/BER	EWS/ERG	66.8
IOR/RCH	EWS/FLI-1 TYPE 2	9.6
IOR/CAR	EWS/ERG	52.0
IOR/BRZ	EWS/FLI-1 TYPE 1	11.6
IOR/CLB	EWS/FLI-1 TYPE 1	13.7
IOR/NGR	EWS/FLI-1	6.9

ES, conventional Ewing's sarcoma; Askin, Askin's tumor; PNET, peripheral neuroectodermal tumor.

A

B

C

Figure 6. Detection of the CCN3 protein with the K19M antibody by Western blotting (**A**) and immunohistochemistry (**B**, TC-71 cells; **C**, 6647 cells).

Figure 7. RT-PCR analysis of frozen tissue samples from giant cell tumors (GCT), chondroblastoma (CBL), chondrosarcoma (CSA), osteosarcoma (OSA), Ewing's sarcoma (EW), and rhabdomyosarcoma (RH). Methods for reverse transcription of mRNA and PCR amplification of cDNA fragments were described in Materials and Methods. Amplification products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

exception of bone Ewing's sarcoma. The expression of CCN3 was high in the osteoclast-like, multinucleated giant cells featuring giant cell tumors and infiltrating chondroblastomas.

In the cartilage tumors, the expression of this protein was lower in the undifferentiated, mixoid component of the tumor and extremely high in the cartilaginous, differentiated areas, indicating an association between CCN3 expression and cartilage differentiation. This is in agreement with findings obtained in normal tissues.¹⁷ In fact, *in situ* hybridization and immunohistochemistry performed on developing chicken and human embryos have revealed a strong expression of *ccn3* RNA and proteins at sites of chondrogenesis.^{16,17} Moreover, by using mesenchymal cells that are able to undergo chondrocytic differentiation *in vitro*, it was possible to establish that CCN3 and CTGF were required at late stages of the chondrogenesis/osteogenesis differentiation process, whereas CYR61 was required at early stages. Whether the differential expression of these three CCN genes is temporally related remains to be established.

Rhabdomyosarcoma expressed high amounts of CCN3, either in cell lines and clinical samples. The expression was particularly evident in the most differentiated cells, supporting the existence of an association between CCN3 expression and tumor differentiation in rhabdomyosarcoma. These findings reflect previous data obtained in normal tissues. Muscle was found to be the

Table 3. Expression of CCN3 in Musculoskeletal Tumors Analyzed on Paraffin-Embedded Sections Using the Rabbit Polyclonal Antibody K19M

	Number of cases	Number of positive cases (%)	Relative score		
			+	++	+++
Giant cell tumor	8	5* (63)	3	0	2
Chondroblastoma	2	2 (100)	0	1	1
Chondrosarcoma central	7	7 (100)	2	2	3
Peripheral	2	2 (100)	0	0	2
Rhabdomyosarcoma embryonal	6	6 (100)	2 [†]	2	2
Alveolar	4	4 (100)	0	0	4
Osteosarcoma Primaries	9	9 (100)	0	2	7
Metastases	4	4 (100)	0	0	4
Ewing's sarcoma	9	2 (22)	0	0	2

*The positivity was referred to the mononuclear cellular component. Osteoclast-like, giant cells are highly positive (+++) in all of the eight samples here analyzed.

[†]In general, expression was more varied in the embryonal group than in the alveolar group. Ovoid shaped, more differentiated cells stained +++.

predominant mesodermal component expressing CCN3 in human embryos and myotubes and fusing myoblasts were identified as major sites of CCN3 expression in skeletal muscle.¹⁷

In osteosarcoma, CCN3 was expressed in all of the tissue samples and in the 61% of the cell lines. Interestingly, in osteosarcoma cell lines, we observed an inverse correlation between expression of CCN3 and alkaline phosphatase, an early marker of osteoblastic differentiation²⁹ that was found to be associated with a loss of aggressiveness of osteosarcoma cells.^{30,31} Based on these findings and on the consideration that MAV can also induce osteopetrosis, an abnormal proliferation of osteoblasts leading to severe bone diseases, it is tempting to speculate that CCN3 may play a role in sustaining the growth of osteoblast-like cells.

Ewing's Sarcoma and CCN3: A Potential Prognostic Indicator of Metastatic Potential

In contrast with the other mesenchymal tumors here examined, Ewing's sarcoma, the second most frequent tumor of bone, showed the expression of CCN3 protein only in the minority of the cases. This could reflect the different origin of Ewing's sarcoma with respect to the other musculoskeletal tumors. The normal counterpart of Ewing's sarcoma is still unknown and this tumor has been the metaphorical "Holy Grail" of pathology in the quest to establish its histogenesis. Although different hypothesis have been raised during the last decades,³² passing through the suggestion of an hematopoietic origin to the idea of a neural origin, the current prevalent opinion is that Ewing's sarcoma derives from a pluripotential uncommitted mesenchymal cell that can variably present neural differentiation, epithelial features, as well as mesenchymal characteristics. The very undifferentiated nature of the Ewing's sarcoma precursor may be responsible for the lower rate of overexpression of CCN3. Whether or not the subgroup of Ewing's sarcoma samples overexpressing CCN3 identifies a specific pathological variety of this neo-

plasm will be the subject of forthcoming studies. In this article, we report on a prognostic significance of CCN3 expression in Ewing's sarcoma. The expression of this protein is, in fact, significantly associated with a higher risk of developing lung and/or bone metastases. Although these findings need to be confirmed in a larger series of patients, they are extremely interesting and promising because CCN3 may be the first biological marker of prognosis for Ewing's sarcoma patients.²⁵ Based on the observation that in Ewing's sarcoma cell lines the expression of CCN3 was found only in those cell lines carrying a type 2 EWS/FLI-1 or a EWS/ERG fusion transcripts, molecular markers associated with worse outcome,^{33,34} it is tempting to speculate about a possible association between these two parameters. A future parallel analysis of CCN3 and the type of fusion transcripts in Ewing's sarcoma clinical samples will settle this question.

In conclusion, CCN3 protein is generally expressed in the cells of the musculoskeletal system, indicating that this protein may play a role both in normal and pathological conditions. However, the regulation of CCN3 expression varies in the different neoplasms and depends on the type of cells. An association between CCN3 expression and tumor differentiation was observed in rhabdomyosarcoma and cartilage tumors, whereas in pronounced contrast and apparent contradiction with these observations, the expression of CCN3 correlated with the metastatic potential of tumor cells in the case of Ewing's sarcoma. Therefore, it seems that the expression of CCN3 is altered in many tumors and that high amounts of the protein could be associated with either differentiation (Wilms' tumor, glioblastoma, neuroblastoma, rhabdomyosarcoma, and chondrosarcoma) or with increased proliferation and metastases (Ewing's sarcoma, prostate cancer, and renal cell carcinoma).¹⁷ This complex and conflicting situation is common to other members of the CCN genes and likely reflects the complexity of functions of these proteins. We believe that additional investigations are required to fully appraise the biological role of CCN3 in tumor progression and malignancy.

Figure 8. Immunohistochemical patterns of CCN3 expression in musculoskeletal tumors. **A:** Giant cell tumor. **B:** Chondroblastoma. Osteoclast-like, multinucleated giant cells stained clearly positive for NovH. **C:** Grade III central chondrosarcoma. **D:** Grade II central chondrosarcoma, a cartilaginous area. **E:** Alveolar rhabdomyosarcoma. **F:** Embryonal rhabdomyosarcoma. **G:** Primary osteosarcoma. **H:** Metastatic osteosarcoma. **I:** A representative example of a negative primary Ewing's sarcoma. **J:** A representative example of a positive primary Ewing's sarcoma. Original magnifications, ×400.

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