available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Short Communication

Expression of transcription factor Yin Yang 1 in human osteosarcomas

Filomena de Nigris[a], *Chiara Botti*[a], *Anna de Chiara*[b], *Raffaele Rossiello*[c], *Gaetano Apice*[b], *Flavio Fazioli*[b], *Carmela Fiorito*[a], *Vincenzo Sica*[a], *Claudio Napoli*[a,*]

[a]*Department of General Pathology, Division of Clinical Pathology, 1st School of Medicine, II University of Naples, Italy*
[b]*National Institute of Tumours, Divisions of Human Pathology and Oncology, Fondazione Pascale, Naples, Italy*
[c]*Division of Human Pathology, 1st School of Medicine, II University of Naples, Italy*

ARTICLE INFO

Article history:
Received 18 April 2006
Accepted 7 June 2006

Keywords:
YY1
Osteosarcoma
Transcription factor

ABSTRACT

The transcription factor Yin Yang 1 (YY1) is known to be present in some human cancer cell lines and its expression correlates with immune-mediated apoptosis. By using Western blot analysis, we have shown that the YY1 protein is strongly expressed in human osteosarcoma cells and localised mainly in the nucleus. Moreover, by using immunohistochemistry and RT-PCR techniques, we have analysed the expression of YY1 protein in biopsies from human osteosarcomas. The YY1 protein was not detectable by immunohistochemistry in osteoid tissue. However, its expression was restricted to osteosarcoma tissues. These data were confirmed by densitometric analysis of RT-PCR for YY1 expression. Thus, YY1 gene activation appears to be an early event in the process of osteoblastic transformation and its detection may represent, together with the analysis of other established markers, a useful diagnostic tool in human osteosarcomas.

1. Introduction

Osteosarcoma is the most common primary malignant bone tumour in children and adolescents.[1–3] Although osteosarcoma is primarily a disease of adolescents and young adults, a different type of osteosarcoma linked to Paget's disease occurs in older adults. The outcome for patients with osteosarcomas is poor with a 2-year overall survival rate of 15–20% following surgical resection, chemotherapy and radiotherapy.[2,4,5] The most frequent site for metastasis is the lung.[6] However, metastasis can also occur in other bones and soft tissue.[6–9] Death from osteosarcoma is usually the result of progressive pulmonary metastasis with respiratory failure due to widespread disease.[1,10,11]

Increasing the understanding of the basic biology of osteosarcoma is a priority for tumour prognosis as well as for providing indications of possible tumour targets for selective therapy. The transcription factor Yin Yang 1 (YY1) is a multifunctional DNA binding protein and an ubiquitously expressed member of a polycomb group of proteins.[12–14] The transcription factor YY1 is known to be present in some human cancer cell lines and its expression correlates to immune-mediated apoptosis.[15–20] Here, we demonstrate for the first time that the transcription factor YY1 is selectively expressed in human osteosarcoma cells and biopsies. The RT-PCR analysis confirmed the data obtained with immunohistochemistry.

* *Corresponding author:* Tel.: +39 081 5665681; fax: +39 081 292399.
E-mail address: claudio.napoli@unina2.it (C. Napoli).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2006.06.008

2. Materials and methods

2.1. Tissue samples

Tissue sections from 10 osteosarcoma tissues for immunohistochemistry were obtained from routinely processed, paraffin-embedded samples obtained both from the Division of Human Pathology of the National Institute of Tumours and the Division of Human Pathology, 1st School of Medicine of the II University of Naples, Italy. The study was approved by the Local Ethical Committee. RNA for RT-PCR was extracted from paraffin blocks on 32 samples including samples before and after chemiotherapy obtained from the National Institute of Tumours of Naples. The age of patients was between 15 and 20 years old.

2.2. Osteosarcoma and normal bone cell lines

The human osteosarcomas SAOS and U2OS and normal osteoblastic 7F2 cell lines were derived from the ATCC catalogue. They were incubated at 37 °C for 7 days in a humidified atmosphere of 95 °C air and 5% CO_2. The incubation medium (DMEM, α MEM GIBCO) was supplemented with 10% FBS, penicillin/streptomycin and glutamine, as described.[21]

2.3. Western blot analysis

Samples containing protein extracts from SAOS-2 and 7F2 cells were analysed by Western blot, as described.[21,22] For isolation of nuclear and cytoplasm proteins, we followed the procedure described previously in detail.[21] Briefly, 10^7 cells were resuspended in 1 ml of ice-cold buffer, 10 mM Hepes, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml pepstatin, 200 mM NaF, on ice for 5 min, centrifugated at 1200 rpm for 5 min, resuspended in 1 ml of the same buffer and incubated in ice for 20 min, then homogenised. The cytosolic fraction was harvested after centrifugation at 1200 rpm for 30 min. Nuclei were obtained by overlaying the homogenised cell solution on a 50% sucrose cushion, followed by centrifugation at 3000 rpm for 1 min and resuspension of the nucleus pellet in sample buffer.

Approximately 30 μg of nuclear protein and 30 μg of cytoplasm protein extracts were separated by 10% SDS–PAGE.[22] The gel was transblotted onto a nitro-cellulose membrane, blocked with 10% milk powder in Tris–buffered saline (pH 7.4) with 0.1% Tween 20 (TBS-T) overnight, and incubated with YY1 antibody purchased from Santa Cruz Biotechnology, as described.[21,22] After five washes with TBS-T, the signal was detected with the aid of a chemiluminescence kit (Amersham Pharmacia enhanced chemiluminescence kit). The same filter was stripped and reprobed with cyclin A antibodies H-432 (Santa Cruz) to normalise the nuclear protein fractions and with mouse monoclonal antibody against γ-tubulin protein T6557 (Sigma) for cytoplasm proteins.

2.4. Immunohistochemistry

Ten osteosarcoma and 10 normal bone tissues from 5 to 6 mm thick paraffin-embedded samples were deparaffinised and then placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min and then washed in PBS before immunoperoxidase staining. Slides were then incubated overnight at 4 °C in a humidified chamber with the primary antibodies diluted 1:100 in PBS and subsequently incubated, first with biotinylated goat antirabbit IgG for 20 min (Vectostain ABC kits; Vector Laboratories) and then with premixed reagent ABC (Vector) for 20 min. The immunostaining was performed by incubating slides in diaminobenzidine (Dako) solution containing 0.06 mM diaminobenzidine and 2 mM hydrogen peroxide in 0.05% PBS (pH 7.6) for 5 min, and after chromogen development, slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips using a permanent mounting medium (Permount). Micrographs were taken on Kodak Ektachrome film with a photo Zeiss system. The antibodies used in this study were rabbit polyclonal raised against the full length of YY1 protein (sc281 Santa Cruz).

The specificity of the reaction was checked by the lack of tissue immunoreactivity after incubation with molar excess of the specific YY1 recombinant *Escherichia coli* protein (sc-4125 Santa Cruz). Immunostained samples were blindly read by two independent investigators and repeated three times.

2.5. Immunofluorescence

Cells were grown on coverslips and were then fixed in paraformaldehyde for 5 min and stained with YY1 antibodies (sc-281, 1:100). The secondary antibodies used were anti-rabbit Alexa 594, (1:800). Preparations were viewed in a Leica confocal TCSS82 microscope and in a Zeiss microscope Axophoto.

2.6. RT-PCR analysis of YY1 expression

RNA was extracted from paraffin-embedded blocks on 22 osteosarcoma biopsies with different histological phenotypes from different cases and 10 normal tissue sections. RNA extraction was performed as described.[23] Briefly, single 6–8 mm tissue sections, cut from paraffin blocks, were stirred overnight in 1.5 ml tubes with 1 ml of xylene. After centrifugation, the pellet was washed with 0.5 ml of ethanol and air-dried. The dried pellet was resuspended in 1 ml of RNagent (Promega) and extracted following the manufacturer's instruction.[23] One-fifth of RNA of total RNA, digested with DNase, was reverse transcribed using random exanucleotides as primers (100 mM) and 12 U of AMV reverse transcriptase (Life Technologies, Inc.), and subsequent PCR amplification was performed as reported previously. Two hundred nanograms of cDNA was amplified in a 25 μl reaction mixture containing *Taq* DNA in polymerase buffer, 0.2 mM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 0.4 mM of each primer, and 1 U of *Taq* DNA polymerase (Perkin–Elmer). The PCR amplification was performed for 30 cycles (94 °C for 30 s, 55 °C for 2 min, and 72 °C for 2 min). The specific primers for YY1 were forward, 5′-ATGGCCTCGGGCGACACCCT-3′; and reverse, 5′-GTCGTCGTCCTCCTCCTCCT-3′, corresponding to the nucleotides 481–500 and 611–630, respectively. Amplification of contaminating genomic DNA was excluded by control experiments in the absence of reverse transcriptase. In addition, a set of primers specific for the constitutively expressed enzyme GAPDH were added to each reaction after 20 cycles of PCR to serve as internal control for the amount of cDNA tested. The GAPDH-specific primers were: forward, 59-ACA-

TGTTCCAATATGATTCC-39 corresponding to the nucleotides 194–214; and reverse 59-TGGACTCCACGACGTACTCAG-39 corresponding to the 336–356 nucleotides.

The product of the reaction was analysed on a 2% agarose gel and then transferred by electroblotting to GeneScreen plus nylon membrane (Du-Pont, Boston, MA). DNA was fixed to the membranes by air drying, UV cross-linking and was hybridised with YY1 probe. The relative level of YY1 expression was assessed in comparison with the level of GAPDH in the same sample by PhosphorImager (Perkin–Elmer).

3. Results

3.1. *Western blot analysis of proteins overexpressed in osteosarcoma cell lines*

We analysed, by Western blot, the expression level and distribution nucleus/cytosol of YY1 protein. We have fractionated the protein extracts from osteosarcoma cell lines Saos-2, U2OS and normal osteoblastic cells (7F2). Fig. 1 shows a representative gel in which 30 μg of nuclear protein extract and 30 μg of cytosol proteins were loaded and analysed with the YY1 antibody. Results revealed a strong expression of YY1 protein in osteosarcoma cell lines compared to normal, with a predominant localisation in the nucleus (10/1 nucleus *versus* cytosol). To show that the nuclear and cytosol proteins from different samples were equally loaded, the same blot was hybridised with cyclin A and γ-tubulin antibodies; the lower panel shows the densitometric analysis. Data showed that the protein YY1 was increased by 10-fold in osteosarcoma samples compared to the control and 90% of the protein was localised in the nucleus. Moreover, we analysed YY1 subcellular localisation by immunofluorescence. Fig. 1 (panel B) shows a representative immunofluorescence performed on Saos-2 cells by using the same antibody as for Western blot. As indicated, the nuclei (stained with Hoechst) were positive to YY1 antibody. The IgG was used as control of specificity and the merge represents the overlap of YY1 and Hoechst staining.

3.2. *Immunohistochemical analysis of YY1 expression*

We analysed 10 different osteosarcoma samples and 10 normal bone controls. All osteosarcomas analysed ($n = 10$) were positive for YY1 (from panels D to H); no immunostaining was observed in normal tissue (panels A and B). Fig. 2 shows a panel of immunohistochemistry staining among our samples. This result is consistent with the published data that showed YY1 protein change localisation translocating from the cytoplasm to the nucleus during the DNA synthesis.[24] Our preliminary data indicated that the staining intensity and frequency measures for both nucleus and cytoplasm expression were higher in neoplastic tissue compared to normal tissue with 60% of nuclei *versus* cells stained.

3.3. *RT-PCR analysis of YY1 expression*

To quantify the expression level of the YY1 gene expression and validate the immunohistochemical data, we analysed 32 samples for the YY1 mRNA levels by a semiquantitative RT-PCR assay. Fig. 3 shows a representative RT-PCR analysis.

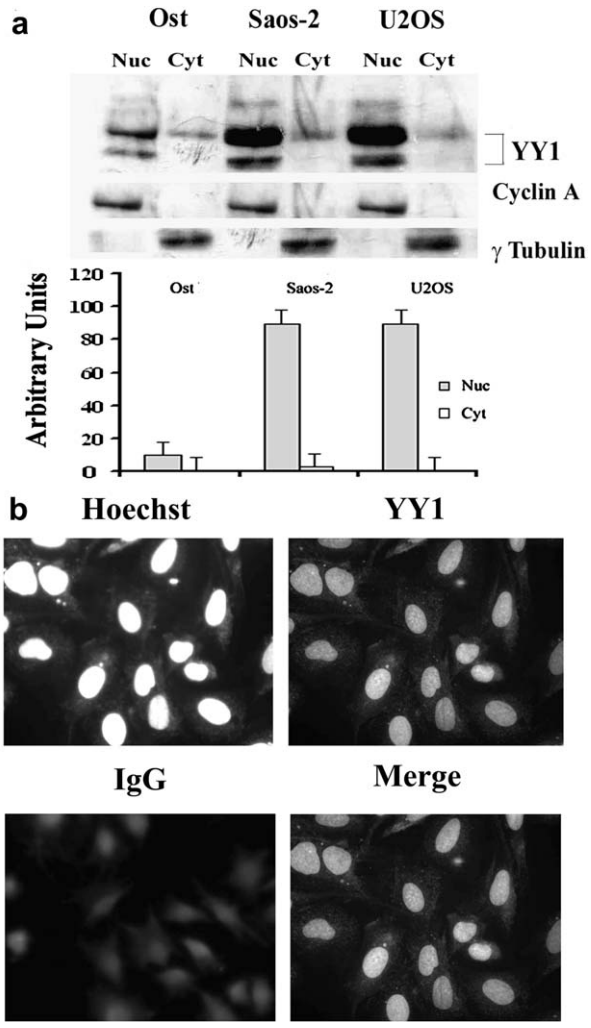

Fig. 1 – (a) Western blot of YY1 expression in osteosarcoma and normal bone cells. Lanes 1 and 2, nuclear and cytoplasm proteins from normal cells; Lanes 3 and 4, nuclear and cytoplasm proteins from osteosarcoma Saos-2 cell; Lanes 5 and 6, nuclear and cytoplasm proteins from U2OS osteosarcoma cells. Densitometric analysis was performed by a PhosphoImager and represents the median values of two different experiments. (b) Cellular localisation of YY1 protein in osteosarcoma cells (SAOS-2) by immunofluorescence. (A) Nuclei stained by Hoechst; (B) immunofluorescence for YY1 protein with the same antibody used in Western blot analysis; (C) negative control cells stained with IgG; (D) merged imaging between Hoechst and YY1 immunostaining.

As shown, the normal bone tissue (Fig. 3, lanes 4 and 5) expressed at very low level mRNA for YY1, while the tumours showed a 10-fold increase of YY1 expression (lanes 2, 3 and lanes 6–11). The GAPDH amplification was used as an internal control. Densitometric analysis performed on all samples (analysed by RT-PCR) revealed that the mean value of YY1 mRNA expression in osteosarcoma samples was 10-fold higher than normal tissues (Fig. 3, panel B). Metastasis samples ($n = 3$) showed a 12-fold increase, the chondrosarcoma ($n = 3$) fivefold increase, fibrosarcoma threefold and Ewing's tumour ($n = 3$) 10-fold increase, respectively. Table 1 reported the

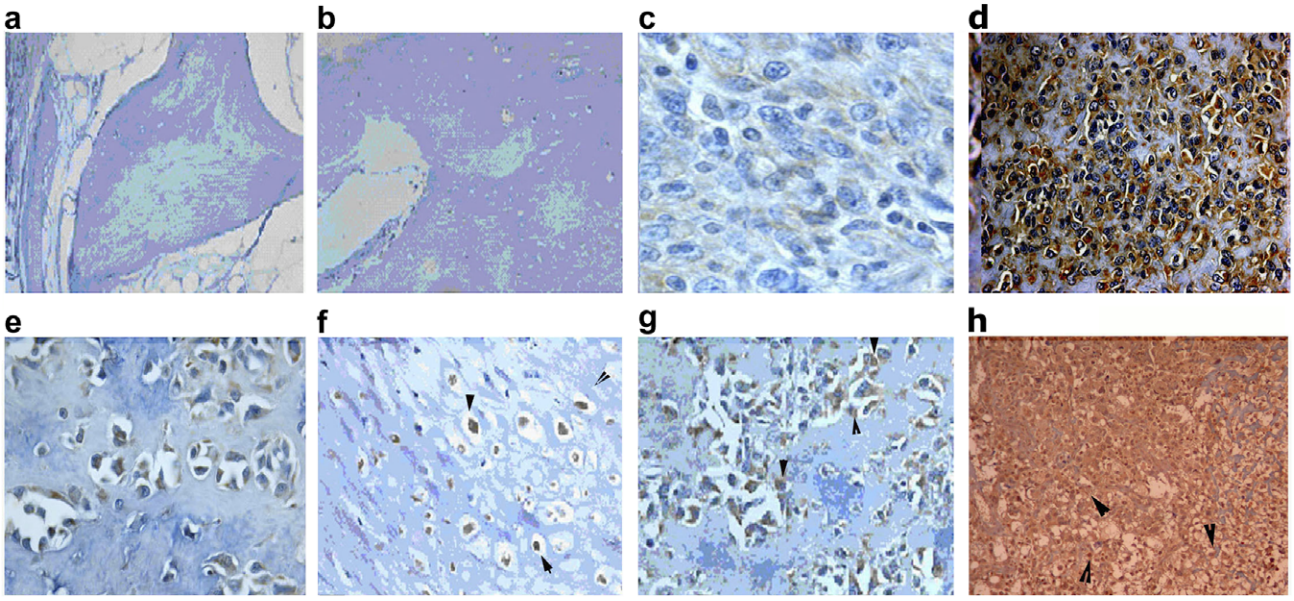

Fig. 2 – Representative immunohistochemistry patterns of YY1 protein expression in osteosarcoma tissues and normal bone (200×). (a,b) Two different normal bone tissues immunostained for YY1 protein; (c) osteosarcoma sample in which the YY1 antibody was preincubated with an excess molar of YY1 recombinant *E. coli* protein negative control; (d) another osteosarcoma sample with immunostaining to YY1 antibodies localised predominantly in the cytoplasm and some nuclei, as indicated by arrows; (e) another representative osteosarcoma with less aggressive phenotype and diffuse staining localised in the cytoplasm; (f) an osteosarcoma sample with high grade phenotype with fine nuclear staining, as indicated by arrows; (g) a different area of the same sample shown in (f) with diffuse staining localised in the cytoplasm and in some nuclei, as indicated by the arrows; (h) osteosarcoma with high grade phenotype with both nuclei and cytoplasm which resulted in a strong immunostaining.

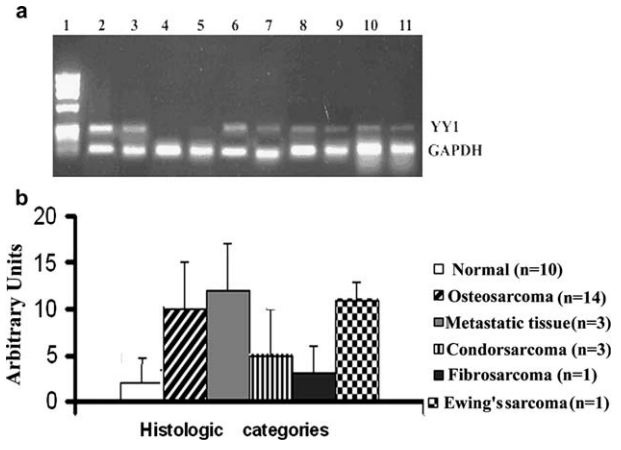

Fig. 3 – (a) RT-PCR analysis of the YY1 gene expression in osteosarcomas. The mRNA was extracted from normal and osteosarcoma tissues and amplified by RT-PCR using specific primers (see Section 2). The cDNA was coamplified for GAPDH as the internal control source of RNA. Representative experiment: lane 1, ladder; lanes 2 and 3, two different osteosarcoma samples; lanes 4 and 5, two samples of normal bone tissues; lanes 6–11, other osteosarcoma samples. This experiment was repeated three times. (b) Densitometric analysis of YY1 mRNA (RT-PCR products) expressed in all tissues samples ($n = 32$). Samples were divided into histologic categories and each sample was normalised for GAPDH.

Table 1 – YY1 expression by immunohistochemistry and RT-PCR in human bone tumours

Histological diagnosis	No. of positive case/no. of cases analysed by RT-PCR (%)
Osteosarcoma ($n = 14$)	13/14 (90)
Chondrosarcoma ($n = 3$)	2/3 (75)
Fibrosarcoma ($n = 1$)	0/1 (0)
Ewing's sarcoma ($n = 1$)	1/1 (100)
Metastasis ($n = 3$)	3/3 (100)
Normal tissue ($n = 10$)	0/10 (0)

Tissue samples were scored as positive for RT-PCR when the amount of YY1 mRNA (measured by densitometric analysis performed on three different experiments) was at least threefold higher than the control.

samples analysed by RT-PCR and divided by histologic categories. Taken together, these data indicate that the YY1 protein was very lowly expressed in normal tissue, whereas tumour tissues showed increased expression levels with higher degrees in more malignant histological phenotypes.

4. Discussion

Here, we have examined the expression pattern of YY1 protein in normal and osteosarcoma tissues. The genesis of this investigation stemmed from results using a cell culture system which demonstrated that YY1 expression is associated with osteosarcoma malignancy. Based on this observation, we

started to examine the expression level of YY1 in human and in malignant bone tissue. We examined YY1 protein expression level using immunohistochemistry and observed an increase in nuclear and cytoplasmic expression in osteosarcomas compared to normal bone tissue. Notably, this represents the first association of YY1 expression with osteosarcoma samples. In normal bone tissue, YY1 was almost absent or in some cases expressed at a very low level in the nucleus (consistent with its activity in transcription regulation). YY1 has been described as a positive or negative regulator of transcription. However, the exact molecular mechanism of action is currently unknown.[13,14] In a very recent study carried out on prostate carcinomas, YY1 protein was present in both the nucleus and in the cytoplasm. In 95% of malignant samples, the YY1 protein was detected mainly in the nucleus.[16] Recently, Palko *et al.*[24] reported that YY1 transits from the cytoplasm to the nucleus at various stages of the cell cycle. The mechanism that regulates the migration of YY1 from cytoplasm to the nucleus is unknown but may depend on nuclear localisation signals and the specific protein signals. It is interesting to consider that the high correlation of expression of YY1 in both nuclear and cytoplasmic compartments in malignant cells may contribute to dysfunction of YY1 activity. The results that we obtained by RT-PCR analysis confirmed the data obtained by immunohistochemistry. The expression of YY1 mRNA in chondrosarcoma and in fibrosarcoma samples was the same as that observed in normal tissue. We considered chondrosarcoma and fibrosarcoma negative for YY1 mRNA. However, further studies in large samples of chondrosarcoma and fibrosarcoma will specifically address this issue. In conclusion, YY1 gene activation appears to be an early event in the process of osteoblastic transformation and its detection may represent, together with the analysis of other markers, a useful tool in the diagnosis of human osteosarcomas.

Conflict of interest statement

None declared.

Acknowledgements

We thank Giuseppina Liguori for technical help. This study was supported by research grants from Regione Campania (L5/2003 and 2004) and 'Ricerca di Ateneo 2005' from the II University of Naples.

REFERENCES

1. Link MP, Eilber F. Pediatric oncology: osteosarcoma. In: Pizzo DG, Poplack DG, editors. *Principles and practice of pediatric oncology*. Philadelphia: Lippincott; 1989. p. 689–711.
2. Weinfeld MS, Dudley Jr HR. Osteogenic sarcoma: a follow-up study of the ninety-four cases observed at the Massachusetts General Hospital from 1920 to 1960. *Am J Orthop* 1962;**44**:269–76.
3. Dahlin DC, Coventry MB. Osteogenic sarcoma. A study of six hundred cases. *J Bone Joint Surg Am* 1967;**49**:101–10.
4. Friedman MA, Carter SK. The therapy of osteogenic sarcoma: current status and thoughts for the future. *J Surg Oncol* 1972;**4**:482–510.
5. Marcove RC, Mike V, Hajek JV. Osteogenic sarcoma under the age of twenty-one. A review of one hundred and forty-five operative cases. *J Bone Joint Surg Am* 1970;**52**:411–23.
6. Fitzgerald Jr RH, Dahlin DC, Sim FH. Multiple metachronous osteogenic sarcoma. Report of twelve cases with two long-term survivors. *J Bone Joint Surg Am* 1973;**55**:595–605.
7. Kaste SC, Pratt CB, Cain AM. Metastases detected at the time of diagnosis of primary pediatric extremity osteosarcoma at diagnosis: imaging features. *Cancer* 1999;**86**:1602–8.
8. Link MP, Goorin AM, Miser AW. The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. *N Engl J Med* 1986;**314**:1600–6.
9. Jeffree GM, Price CH, Sissons HA. The metastatic patterns of osteosarcoma. *Br J Cancer* 1975;**32**:87–107.
10. Rosen G, Forscher CA, Mankin HJ. Neoplasms of bone and soft tissue: bone tumors. In: Bast RCJ, Kufe DW, Pollock RE, et al., editors. *Cancer medicine*. 5th ed. Hamilton, Canada: B.C. Decker, Inc.; 2000. p. 1870–902.
11. Meyers PA, Gorlick R. Osteosarcoma. *Pediatr Clin North Am* 1997;**44**:973–89.
12. Shi Y, Seto E, Chang LS, Shenk T. Transcriptional repression by YY1, a human GLI-Kruppel- related protein, and relief of repression by adenovirus E1A protein. *Cell* 1991;**67**:377–88.
13. Shi Y, Lee JS, Galvin KM. Everything you have ever wanted to know about Yin Yang 1. *Biochim Biophys Acta* 1997;**1332**:F49–66.
14. Gordon S, Akopyan G, Garban H, Bonavida B. Transcription factor YY1: structure, function, and therapeutic implications in cancer biology. *Oncogene* 2006;**23**:1125–42.
15. Brankin B, Skaar TC, Brotzman M, Trock B, Clarke R. Autoantibodies to the nuclear phosphoprotein nucleophosmin in breast cancer patients. *Cancer Epidemiol Biomarkers Prev* 1998;**7**:1109–15.
16. Seligson D, Horvath S, Huerta-Yepez S, et al. Expression of transcription factor Yin Yang 1 in prostate cancer. *Int J Oncol* 2005;**27**:131–41.
17. Vega ML, Jazirchi AR, Huerta-Yepez S, Bonavida B. Rituximab-induced inhibition of YY1 and Bcl-xL expression in Ramos non Hodgkin's lymphoma cell line via inhibition of NF-KappaB activity: role of YY1 and Bcl-xL in Fas resistance and chemoresistance, respectively. *J Immunol* 2005;**175**:2174–83.
18. Bain M, Sinclair J. Targeted inhibition of the transcription factor YY1 in an embryonal carcinoma cell line results in retarded cell growth, elevated levels of p53 but no increase in apoptotic cell death. *Eur J Cell Biol* 2005;**84**:543–53.
19. Garban HJ, Bonavida B. Nitric oxide inhibits the transcription repressor Yin-Yang 1 binding activity at the silencer region of the Fas promoter: a pivotal role for nitric oxide in the up-regulation of Fas gene expression in human tumor cells. *J Immunol* 2001;**167**:75–81.
20. Gronroos E, Terentiev AA, Punga T, Ericsson J. YY1 inhibits the activation of the p53 tumor suppressor in response to genotoxic stress. *Proc Natl Acad Sci USA* 2004;**101**:12165–70.
21. de Nigris F, Youssef T, Ciafre S, et al. Evidence for oxidative activation of c-Myc-dependent nuclear signaling in human coronary smooth muscle cells and in early lesions of Watanabe heritable hyperlipidemic rabbits: protective effects of vitamin E. *Circulation* 2000;**102**:2111–7.
22. de Nigris F, Crutti J, Morelli C, et al. Isolation of a SI-like gene, Sir-T8, that is overexpressed in thyroid carcinoma cell lines and tissues. *Br J Cancer* 2002;**86**:917–23.
23. Chiappetta G, Tallini G, De Biasio MC, et al. FRA-1 expression in hyperplastic and neoplastic thyroid diseases. *Clin Cancer Res* 2000;**6**:4300–6.
24. Palko L, Bass HW, Beyrouthy MJ, Hurt MM. The Yin Yang-1 (YY1) protein undergoes a DNA-replication-associated switch in localization from the cytoplasm to the nucleus at the onset of S phase. *J Cell Sci* 2004;**117**:465–76.