

MDM2 Gene Amplification in Metastatic Osteosarcoma¹

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Abstract

The human homologue of the murine double minute 2 gene (*MDM2*), a p53-binding protein which may act as a regulator of p53 protein function, has recently been cloned. Initial studies of this gene in a variety of human tumors have shown frequent gene amplification in most types of sarcomas, including osteosarcomas. Amplification of the *MDM2* gene may produce a functional inactivation of the p53 protein. To examine possible clinical or pathological correlates of *MDM2* gene amplification in osteosarcoma, we studied 28 specimens on 26 patients with high grade osteosarcoma (16 primary, 11 metastatic, and 1 local recurrence) for *MDM2* gene amplification by Southern blot analysis, using two *MDM2* complementary DNA probes isolated by polymerase chain reaction. Four specimens (14%) showed amplification, including 3 metastases and 1 local recurrence. None of the primary osteosarcoma specimens had detectable *MDM2* gene amplification. None of the specimens tested showed *MDM2* gene rearrangement. In the present series, *MDM2* gene amplification was detected significantly more frequently in metastatic or recurrent osteosarcomas than it was in primary osteosarcomas ($P = 0.02$). Our data suggest that *MDM2* gene amplification may be associated with tumor progression and metastasis in osteosarcoma. Further investigation is warranted on the potential clinicopathological correlates of *MDM2* gene amplification in osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignant tumor of bone. Approximately 60% of cases are pediatric. Ten to 20% of patients have metastases detectable at diagnosis. Prognostic factors among patients with primary tumors include primary site, histological response of tumor to preoperative chemotherapy, and serum lactate dehydrogenase and alkaline phosphatase at diagnosis (1). Additional markers identifying patients likely to relapse would be useful, since even in the most current treatment protocols, at least 25% of pediatric patients with extremity primaries develop recurrent or metastatic disease within 5 years (1).

The human homologue of *MDM2*,⁵ a p53-binding protein, has recently been cloned (2). The product of this gene appears to act as a regulator of p53 protein function, in both mice and humans (2, 3). The gene was originally isolated from a tumorigenic mouse fibroblast cell line containing double minutes, a cytogenetic hallmark of gene amplification (4). Overexpression of this gene induces tumorigenicity in murine cell lines (5). High levels of the *MDM2* gene product may result in functional inactivation of the p53 protein, much like some viral products such as adenovirus protein E1B, SV40 T-antigen, or human papilloma virus protein E6, which can also bind and inactivate the p53 protein (6). Hence, *MDM2* amplification may provide a mechanism of p53 inactivation distinct from genomic alterations of p53.

Initial studies of the *MDM2* gene in a variety of human tumors have shown frequent gene amplification in most types of sarcomas, including 3 of 11 osteosarcomas (2). To examine possible clinical or pathological correlates of *MDM2* gene amplification in osteosarcoma, we studied 28 specimens of high grade osteosarcoma for *MDM2* gene amplification.

Materials and Methods

The study material consisted of 28 specimens of high grade osteosarcoma obtained from 26 patients who underwent surgery at Memorial Sloan-Kettering Cancer Center. Cases were selected on the basis of availability of frozen material for study and on the absence of extensive chemotherapy-induced tumor necrosis, *i.e.*, Grade III or IV chemotherapy effect (90–100% necrosis) (7). The specimens included 16 primary, 11 metastatic, and 1 locally recurrent tumors. Among the 26 patients, there were 14 pediatric cases (ages <22 years) and 12 adult cases (ages >21 years). Basic clinical data are presented in Table 1.

Two patients provided two specimens each. In one patient (samples OS-5 and OS-8), the second specimen was from an excision of residual tumor at the primary site. In the other case (samples OS-11 and OS-13), the second specimen was a lung metastasis. None of the patients had a history of retinoblastoma, although two patients had prior primary malignant tumors. In case OS-7, the patient had a pelvic osteosarcoma resected 6 years earlier, with no recurrent or residual disease. His ulnar osteosarcoma was considered a new primary on clinical and histological grounds. In case OS-31, a myxoid liposarcoma of the thigh was resected 2 years prior to the primary diagnosis of osteosarcoma of the proximal tibia, 6 years prior to the recurrence of this osteosarcoma from which our specimen was obtained.

Two cDNA probes were synthesized by polymerase chain reaction using normal human liver cDNA (Clontech, Palo Alto, CA) as a template. The 585-base pair *MDM2A* probe spanned nucleotides 650 to 1214 of the published cDNA sequence (2) and was amplified using the following primers: 5'-AATCATCGGACTCAGGTACA-3'; and 5'-GTACGCTAAGGA-AATTCAGG-3'. The 476-base pair *MDM2B* probe spanned nucleotides 1208 to 1631 of the cDNA (2) and was amplified using the primers 5'-GCTGACTATTGGAAATGCAC-3' and 5'-TCGACCTTGACAAATCACAC-3'. The identity of the probes was supported by their derivation from single polymerase chain reaction products of the correct size in agarose gels and confirmed by their hybridization exclusively to bands of the expected size in Southern and Northern blots. The two probes were used interchangeably.

Amplification of the *MDM2* gene was studied by Southern blot analysis of *EcoRI*- or *HindIII*-digested DNA. The Southern blot hybridization signal of the *MDM2A* or *MDM2B* probe was compared to that of probe D12S2 (American Type Culture Collection 57181), also located on chromosome 12. A reference probe from the same chromosome was used to control for variations in sample loading and for the effect of nonspecific polysomies of chromosome 12 on the quantitation of gene copy number (8). Signals were quantitated on a Betascope 630 Blot Analyzer (Betagen, Waltham, MA).

Results and Discussion

Northern Blot Analysis. The pattern of *MDM2* RNA expression was examined in a panel of normal human organ-derived mRNAs, including heart, brain, placenta, lung, liver, muscle, kidney, and pancreas. A 5.5-kilobase transcript, corresponding in size to that reported previously (2), was demonstrated, thereby also confirming the identity of the amplified probes (Fig. 1). In this panel of tissues, RNA expression appeared highest in skeletal muscle but was also evident in

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⁵ The abbreviations used are: *MDM2*, murine double minute 2; cDNA, complementary DNA.

liver, lung, and pancreas. In adult mice, among heart, brain, lung, testis, thymus, and muscle, *MDM2* RNA expression appeared highest in testis, thymus, and brain (5). The widespread expression of *MDM2* in both murine and human tissues supports its involvement in basic cellular processes.

Amplification Analysis. Amplification of the *MDM2* gene was present in 4 of 28 osteosarcomas (Fig. 2; Table 1). Sample OS-27 was amplified 18-fold and was a lung metastasis from a 39-year-old female who had a pelvic osteosarcoma resected 4 years earlier. OS-31 showed 7-fold amplification and was a fibroblastic osteosarcoma representing a local recurrence of a juxtacortical osteosarcoma of the proximal tibia resected 4 years earlier. OS-26 and OS-16 were amplified 5- and 4-fold, respectively, and were lung metastases arising

Table 1 Basic clinical and molecular data^a

Unique tumor no.	Age at primary diagnosis (yr)	Sex	Primary site	Source of specimen	<i>MDM2</i> amplification
OS-1	7	M	Humerus	P	1
OS-2	40	M	Tibia	P	1
OS-3	10	F	Femur	P	1
OS-5	83	F	Fibula	P	1
OS-6	7	F	Femur	P	1
OS-7	18	M	Ulna	P	1
OS-8	83	F	Fibula	P	1
OS-11	18	F	Femur	M	1
OS-12	15	M	Femur	M	1
OS-13	18	F	Femur	P	1
OS-14	22	M	Femur	M	1
OS-15	37	M	Femur	P	1
OS-16	31	F	Tibia	M	4
OS-17	15	F	Patella	P	1
OS-18	47	M	Tibia	M	1
OS-19	15	F	Femur	P	1
OS-21	11	M	Tibia	M	1
OS-22	60	M	Tibia	P	1
OS-24	16	F	Clavicle	P	1
OS-25	8	M	Skull	M	1
OS-26	42	M	Humerus	M	5
OS-27	34	F	Ischium	M	18
OS-29	20	M	Ilium	P	1
OS-30	81	F	Tibia	M	1
OS-31	28	F	Tibia	R	7
OS-32	25	F	Ilium	P	1
OS-33	19	M	Tibia	M	1
OS-34	16	M	Femur	P	1

^a P, primary; M, lung metastasis; R, local recurrence.

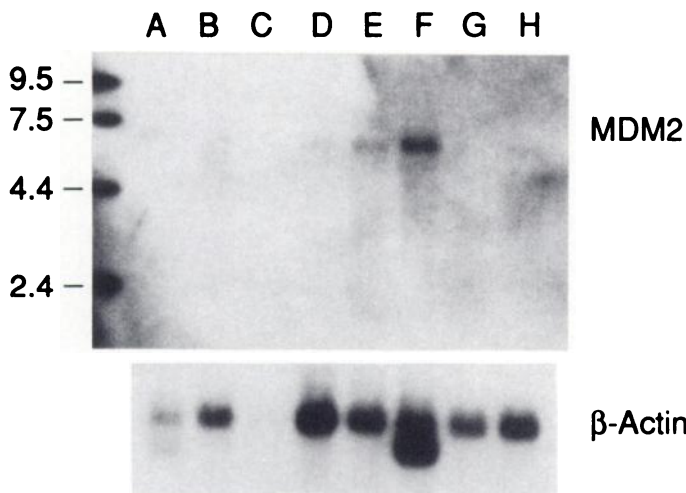


Fig. 1. Northern blot analysis of *MDM2* expression in a panel of normal human tissues (Multiple Tissue Northern Blot; Clontech, Palo Alto, CA). Each lane contains 2 µg of mRNA extracted from: A, heart; B, brain; C, placenta; D, lung; E, liver; F, skeletal muscle; G, kidney; H, pancreas. Left, positions of the bands in the marker lane in kilobases. The *MDM2* probe hybridizes to a 5.5-kilobase transcript. Bottom, control β -actin hybridization confirming adequate loading of all lanes.

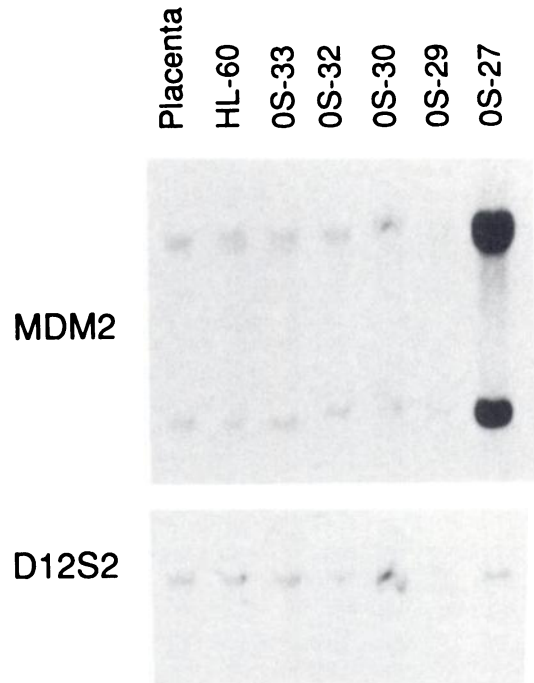


Fig. 2. Amplification analysis of the *MDM2* gene. Quantitative comparison of the Southern blot hybridization signal with the *MDM2A* probe (top) and with the reference probe, D12S2, following rehybridization (bottom) showed 18-fold amplification of the *MDM2* gene in sample OS-27. The remaining samples on this blot, as well as the HL-60 cell line, showed no amplification. Placental DNA was used as the negative control for amplification.

from primaries removed 4 and 5 years earlier, respectively. All 4 specimens with *MDM2* amplification were obtained from adult patients.

In the present series, *MDM2* gene amplification was detected significantly more frequently in metastatic or recurrent tumors than in primaries (0 of 16 versus 4 of 12; Fisher's exact test, $P = 0.02$). The overall percentage of amplified cases in our series (14%) is slightly lower than that reported by Oliner *et al.* (27%; Ref. 2) although the difference does not appear statistically significant (Fisher's exact test, $P = 0.38$). Clinical data such as the proportion of metastatic specimens in their group of 11 osteosarcomas were not provided (2).

In general, the proportion of amplified cases is expected to be lower in clinical specimens because admixed nonneoplastic cells may obscure low level amplification in the tumor cells. For similar reasons, the level of amplification in amplified cases also appears lower in clinical specimens than in cell lines. The necessary exclusion of primary osteosarcomas showing Grade III to IV chemotherapy effect (7) from the present series clearly altered the proportion of primary to metastatic cases and eliminated most of the highly chemosensitive tumors, which represent a favorable prognostic subset (1).

Low level amplification of the *MYC* gene was previously identified in two of the cases in the present series, OS-15 and OS-22, but was not detected in any of the cases shown here to have *MDM2* gene amplification.⁶

The *MDM2* gene has been mapped by somatic cell hybrid analysis to 12q13-14 (2), a region containing two other genes, *SAS* and *gli*, previously found to be amplified in various sarcomas. *SAS*, a gene of unknown function, was isolated from a malignant fibrous histiocytoma by the in-gel renaturation technique and is amplified in some malignant fibrous histiocytomas and liposarcomas (9, 10). The *gli* gene, which codes for a putative DNA-binding zinc finger protein,

⁶ M. Ladanyi, C. K. Park, R. Lewis, S. C. Jhanwar, J. H. Healey, and A. G. Huvos, submitted for publication.

was originally isolated from a glioblastoma and was found to be amplified in one rhabdomyosarcoma and one small cell osteosarcoma (11). Coamplification of several syntenic potential candidate genes is well described in band 11q13 in breast cancer (12). A similar process may affect a region in 12q13-14 containing *MDM2*, *SAS*, and *gli* in sarcomas, although the *gli* and *SAS* genes were not found to be coamplified in the respective cell lines from which they were isolated (9).

The pivotal role of *p53* alterations in osteosarcoma is widely recognized. Strong evidence has been provided by analyses of the *p53* gene and mRNA in osteosarcomas (13, 14) and from the frequent occurrence of osteosarcoma both in transgenic mice expressing mutant *p53* protein and in the Li-Fraumeni syndrome (15, 16). Although not extensively studied, *p53* alterations in many if not most osteosarcomas probably occur in conjunction with retinoblastoma gene inactivation (17-19).

Allelotyping studies suggest that the *p53* gene may be involved in up to 70-75% of osteosarcomas (17, 18). Genetic mechanisms involved in the minority of cases lacking *p53* alterations have until now been unknown. The *MDM2* gene product is known to bind both wild-type and mutant *p53* protein, and this interaction inhibits transactivation mediated by wild-type *p53* protein (3). Hence, *MDM2* amplification may constitute an alternative mechanism of *p53* inactivation. Indeed, preliminary observations suggest that *MDM2* amplification may occur mainly in sarcomas lacking detectable *p53* alterations (2). Since different mutant alleles of *p53* have different biological properties (6), *MDM2* amplification could also offer an added selective advantage to tumors with "weaker" mutant alleles, e.g., mutants for residue 273.

Hence, the study of *MDM2* amplification may help to elucidate genetic mechanisms in osteosarcomas lacking abnormalities of the *p53* gene. Furthermore, the observed amplification of *MDM2* in metastatic and recurrent lesions in the present series suggests that this event may signal or promote tumor progression in some osteosarcomas. In several types of tumors (e.g., neuroblastoma and breast carcinoma), the amplification of specific genes is predictive of a poorer clinical course (8, 12). Further investigation is warranted on the potential usefulness of *MDM2* gene amplification as a prognostic indicator in osteosarcomas and on the relationship between *p53* and *MDM2* gene alterations in this tumor group.

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