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ORIGINAL ARTICLE

Amplification and over-expression of the *MDM2* gene in human soft tissue tumours

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Abstract

Purpose. Amplification of genetic sequences on chromosome 12q13 is frequently found in soft tissue tumours. However, for the *MDM2* gene, over-expression of the MDM2 protein has not always been shown to accompany gene amplification, raising the possibility that amplification of genetic sequences targets alternative genes on chromosome 12q13 for over-expression. To investigate this discrepancy, we have examined 129 soft tissue tumours for amplification of the *MDM2* gene using Southern analysis, and 39 of these tumours were also examined by immunohistochemical staining for MDM2 over-expression.

Results. Gene amplification was identified in 14/114 (12.3%) of the malignant tumours, but was not identified in any of the benign tumours; 21/39 (54%) of the malignant tumours also demonstrated MDM2 over-expression. Within this group the *MDM2* gene was over-expressed in every tumour in which the gene amplification was found, and over-expression in the absence of gene amplification was also found in an additional 10 tumours.

Discussion. These data demonstrate a clear correlation between the presence of *MDM2* amplification and MDM2 over-expression, and provide persuasive evidence therefore that the amplification of genetic sequences on chromosome 12q13 in soft tissue sarcomas targets the *MDM2* gene for over-expression. These data also indicate that alternative mechanisms may contribute to MDM2 over-expression within some tumours.

Key words: MDM2 *gene, amplification, over-expression, soft tissue sarcoma*.

Introduction

A variety of mammalian tumour cells contain amplified copies of genes whose transforming potential is then activated by their concomitant over-expression. In keeping with this, double minute chromosomes and homogeneously staining regions have been identified in malignant fibrous histiocytomas, liposarcomas, and alveolar and embryonal rhabdomyosarcomas.[1–3] A cluster of genes which map to chromosome 12q13 have been found to be amplified and over-expressed in soft tissue sarcomas, and include the *MDM2, GLI, CDK4, GADD153* (CHOP) and *SAS* genes.[4–8]

The *MDM2* gene was originally identified as a dominantly transforming oncogene amplified and over-expressed from double minute chromosomes in the 3T3DM cell line.[9] *MDM2* was subsequently shown to cooperate with *RAS* in the transformation of primary rat embryo fibroblasts, and to be amplified in liposarcomas, malignant fibrous histiocytomas (MFHs) and osteosarcomas.[4] MDM2 has been shown to bind p53 and inhibit p53-mediated transactivation[10] and is itself a target for p53-mediated gene regulation via a *cis*-acting p53 response element in its first intron.[11] Elevated p53 expression results in increased levels of MDM2 mRNA and protein, which in turn binds to and modulates p53 function. It has now also been shown that MDM2 can bind and inhibit the growth regulatory function of RB1[12] and can activate E2F1/DP1 transcription factors which are involved in promoting entry into the S-phase.[13] The oncogenic activity of MDM2 may therefore derive from its ability both

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to repress p53 function and to stimulate the activity of S-phase promoting transcription factors.

A variety of studies have examined *MDM2* amplification and over-expression in soft tissue tumours.[14–17] These studies, together with studies on other tumour types, have demonstrated that MDM2 over-expression as assessed by immunohistochemical staining is frequently found in the absence of *MDM2* gene amplification,[14,18,19] and that MDM2 over-expression may also be absent in tumours in which amplification has been demonstrated by Southern analysis.[14] These latter observations raise the possibility that gene amplification on chromosome 12q13 may target a gene or genes other than *MDM2* for over-expression. We therefore sought to further investigate the role of the *MDM2* gene in soft tissue tumour development by analyzing a large series of malignant and benign tumours for *MDM2* amplification using Southern analysis and scanning densitometry, and MDM2 and p53 over-expression was examined in a subgroup of these tumours using immunohistochemical staining of frozen sections.

Materials and methods

Clinical samples and cell lines

Fresh specimens of primary soft tissue tumours were obtained during surgical resection from the Royal Marsden Hospital, London and Surrey, St Thomas's Hospital, London and the Royal Orthopaedic Hospital, Birmingham. Samples were immediately snap frozen in liquid nitrogen and stored at -70°C until processed. Human cell lines with the exception of RMS[20] were obtained from the American Type Culture Collection (ATCC) and maintained as recommended by their supplier.

Southern analysis

DNA was extracted using a modification of a method described by Steffen and Weinberg.[21] Genomic DNA (10 μg) was digested with a three- to five-fold excess of HindIII or *Eco*RI, subjected to electrophoresis in 0.8% agarose gels and transferred to Hybond-N filters (Amersham) according to the manufacturer's instructions. Filters were hybridized according to published protocols[22] to α-^{32}P-labelled probes prepared using random oligonucleotide primers.[23] Filters were washed at 65°C to a final stringency of 0.1 × SSC, 0.1% SDS.

Amplification of the *MDM2* gene was assessed using a 1.5-kb *MDM2* probe prepared by PCR amplification of reverse-transcribed normal fibroblast RNA using the following primers: GGGAGCTCCTCGCCACCATGGTGAGGAGCAGGCAAATG and GGGGTACCCTCATAGACAGGTCAACTAGGGG. The PCR product was subcloned into the pBluescript vector and characterized by sequencing of its entire length. Following hybridization to the *MDM2* probe, blots were stripped by immersion in 0.1% SDS at 95°C and reprobed with p*DCC*1.0 as a control to correct for differences between tumour samples in DNA loading and Southern transfer.[4] The *DCC* gene probe used was chosen as a control because allele loss at the *DCC* locus is found in only around 10% of soft tissue sarcomas, and homozygous deletion has not been demonstrated.[24] Allele loss without reduplication at the *DCC* locus in tumour DNA would result in a two-fold overestimation of the degree of *MDM2* amplification, and this error will be introduced in fewer than 10% of the tumours examined. The degree of amplification was quantified with a Joyce-Loebel Chromoscan 3 scanning densitometer, using absorbance at 530 nm. To allow for inaccuracies due to the effects of stromal contamination, and for the non-linear response of the radiographic film, only samples showing greater than five-fold amplification were considered to be amplified.

Immunohistochemical staining of tumour specimens for MDM2 and p53 expression

Two anti-MDM2 mouse monoclonal antibodies MDM2 (clone IF2) (David Hill, Oncogene Science Inc.) and SMP14,[25] and the p53 antibody DO1 (Oncogene Science Inc.) were used to stain frozen sections of primary tumour specimens using a standard peroxidase-conjugated streptavidin-biotin method (ABC). Slides omitting the first antibody were used as negative controls in all cases. Two tumours known to be positive for either MDM2 or p53 were included as positive controls in each batch of staining. The degree of immunostaining was graded as follows: (negative) $<20\%$ of cells stained positively, (+) 20–50% of cells stained positively, (++) $>50\%$ of cells stain positively.

Results

One hundred and twenty-nine tumours (124 primary tumours and five cell lines) including 29 leiomyosarcomas, 17 liposarcomas, 16 MFHs, 16 rhabdomyosarcomas, 12 malignant peripheral nerve sheath tumours (MPNSTs), 10 synovial sarcomas, 3 osteosarcomas, 2 fibrosarcomas, 2 chondrosarcomas, 7 other sarcomas, 5 fibromatoses and 10 benign soft tissue neoplasms were analyzed. Greater than five-fold amplification of *MDM2* was detected in 14/114 (12.3%) of the malignant tumours but in none of the fibromatoses or benign tumours. The positive samples included 3/16 (19%) MFHs (STS11, STS41 and STS140), 4/17 (24%) liposarcomas (STS20, STS44, STS61 and STS131), 2/29 (7%) leiomyosarcomas (STS87 and STS320), 2/16 (12.5%) rhabdomyosarcomas (1 embryonal rhabdomyosarcoma STS93 and the alveolar rhabdomyosarcoma cell line RMS), 2/12 (17%) MPNSTs (STS52 and STS102) and a single

Table 1. *Amplification of the* MDM2 *gene in soft tissue tumours*

Tumour type	Number examined	Number. amplified	Percentage amplified
Leiomyosarcoma	29	2	7
MFH	16	3	19
Liposarcoma	17	4	24
Rhabdomyosarcoma	16	2	12.5
MPNST	12	2	17
Synovial sarcoma	10	0	0
Other sarcomas*	14	1	7
Fibromatosis	5	0	0
Benign tumours**	10	0	0

*Includes 3 osteosarcomas, 2 chondrosarcomas, 2 fibrosarcomas (one of which showed *MDM2* amplification), 1 sarcoma NOS, 2 clear cell sarcomas, 1 dermatofibrosarcoma proturberans, 1 post-irradiation spindle cell sarcoma, 1 neuroepithelioma and 1 renal leiomyoblastoma.

**Includes 3 haemangiomas, 3 neurofibromas, 2 neurilemmomas, 1 paraganglioma and 1 angiolipoma.

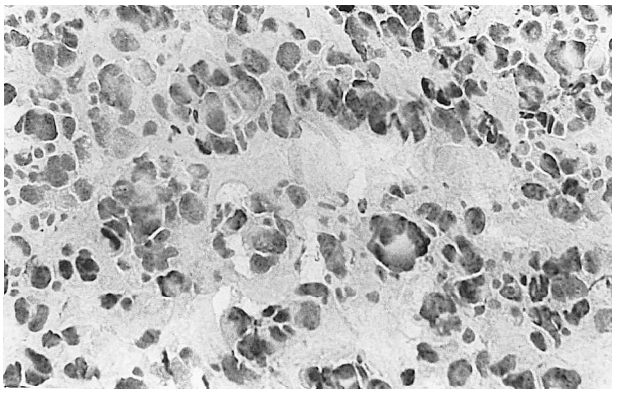

Fig. 2. *Immunohistochemical staining of primary tumours for MDM2 over-expression. Immunohistochemical staining of STS24, a high-grade MFH, using the mouse monoclonal MDM2 antibody MDM2 (IF2) (Oncogene Science). Greater than 50% of the cells show positive staining for MDM2.*

fibrosarcoma (STS49). These results are presented in Table 1, and examples of *MDM2* gene amplification are illustrated in Fig. 1. STS162, a recurrence of the liposarcoma STS44, was not included in the original group of tumours but was subsequently analyzed, and also demonstrated *MDM2* gene amplification.

Thirty-nine of the tumours analyzed for *MDM2* amplification were also examined for MDM2 over-expression by immunohistochemical staining (Table 2). These tumours were examined without knowledge of the *MDM2* gene amplification results, but had been selected to include 11 of the tumours with *MDM2* amplification. This group included 8 liposarcomas, 8 MFHs, 6 leiomyosarcomas, 5 MPNSTs, 3 rhabdomyosarcomas, 3 synovial sarcomas, 2 sarcomas NOS (not otherwise specified), 1 clear cell sarcoma, 1 fibrosarcoma and 2 fibromatoses. Of these, 21/39 (54%) including 5 MFHs, 4 liposarcomas (including STS44 and its recurrence STS162), 3 MPNSTs, 3 rhabdomyosarcomas, 2 leiomyosarcomas, 2 synovial sarcomas, 1 fibrosarcoma and 1 clear cell sarcoma showed positive immunohistochemical staining for MDM2. An example is shown in Fig. 2. All 11 of the tumours in which the *MDM2* gene was amplified stained positively for MDM2 over-expression, and MDM2 over-expression was confirmed in the RMS cell line by Northern analysis. Conversely, the *MDM2* gene was amplified in 0/18 (0%) of the negatively staining tumours. Of the six tumours with strongly positive staining for MDM2 (>50% of cells positive), 5/6 (83%) demonstrated *MDM2* amplification, whereas only 6/15 (40%) of the relatively weakly staining tumours (20–50% cells positive) demonstrated *MDM2* amplification. The level of amplification in weakly staining tumours was, however, comparable to that seen in the more strongly staining tumours. Among five additional tumours, for which data regarding *MDM2* gene amplification were not available, over-expression was identified in two MFHs, STS7 and STS9.

p53 over-expression, as assessed by immunohistochemical staining, was identified in four tumours. In two of these tumours, STS24 and STS102, the *MDM2* gene was also over-expressed, and in one of these tumours, STS102, a malignant peripheral nerve sheath tumour, *MDM2* gene amplification was found.

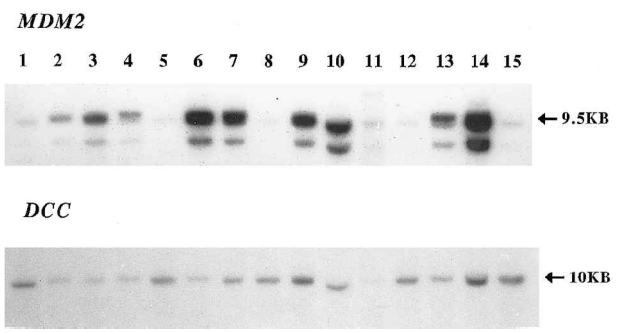

Fig. 1. *Amplification of the* MDM2 *gene in malignant soft tissue tumours. Southern blot analysis of* Eco*RI-digested DNA probed sequentially with p*DCC*1.0 and an* MDM2 *cDNA probe, demonstrating* MDM2 *gene amplification in several tumours. The degree of gene amplification was estimated by using scanning densitometric analysis of the resulting autoradiographs. The loading is as follows: lane 1, normal leucocyte DNA; lane 2, RMS cell line; lane 3, STS93; lane 4, STS102; lane 5, normal leucocyte DNA; lane 6, STS49; lane 7, STS11; lane 8, normal leucocyte DNA; lane 9, STS41; lane 10, STS44; lane 11, STS61; lane 12, normal leucocyte DNA; lane 13, STS140; lane 14, STS162; lane 15, normal leucocyte DNA.*

Table 2. *Amplification and/or over-expression of the* MDM2 *gene in human soft tissue sarcomas*

Tumour	Histology	Grade	Source	*MDM2* gene amplification*	MDM2 over-expression**
STS7	MFH	High	Recurrence	NE	+
STS9	MFH	High	Recurrence	NE	+
STS11	MFH	High	Recurrence	36	+
STS20	Liposarcoma	Low	Primary	7	NE
STS23	Pleomorphic rhabdomyosarcoma	High	Primary	ND	+
STS24	MFH	High	Recurrence	ND	+ +
STS32	Clear cell sarcoma	High	Primary	ND	+
STS34	Leiomyosarcoma	High	Primary	ND	+
STS41	MFH	Low	Recurrence	14	+
STS44	Liposarcoma	High	Recurrence	33	+
STS49	Fibrosarcoma	Low	Recurrence	>50	+ +
STS52	MPNST	High	Primary	6	+
STS56	MFH	High	Recurrence	ND	+
STS61	Liposarcoma	Intermediate	Recurrence	44***	+
STS62	MPNST	High	Primary	ND	+
STS69	Alveolar rhabdomyosarcoma	High	Primary	ND	+
STS87	Leiomyosarcoma	Low	Metastasis	>50	+ +
STS93	Embryonal rhabdomyosarcoma	High	Metastasis	50	+ +
STS102	MPNST	Low	Recurrence	20	+ +
STS114	Synovial sarcoma	High	Primary	ND	+
STS117	Liposarcoma	High	Recurrence	ND	+
STS125	Synovial sarcoma	High	Primary	ND	+
STS131	Liposarcoma	Low	Primary	48	NE
STS140	MFH	High	Primary	34	+
STS162	Liposarcoma	High	Recurrence	28	+ +
STS320	Leiomyosarcoma	High	Primary	>50	NE
RMS	Rhabdomyosarcoma	—	Cell line	14	+ [+]

*Amplification was detected by Southern blot analysis using an *MDM2* cDNA probe spanning the whole open reading frame. Scanning densitometry was used to compare the signal to that obtained with the probe p*DCC1*.0.

**Detected by immunohistochemical staining of frozen sections with the monoclonal antibody MDM2 (Ab-1) (Oncogene Science). Staining was graded (+) 20–50% cells stain positively, (+ +) >50% of cells stain positively.

***Problems with DNA degradation and digestion with this sample impair the accuracy of this result.

[+] Northern analysis.

NE = not evaluated; ND = not detected.

Discussion

This study confirms the observation of amplification of the *MDM2* gene in soft tissue sarcomas which was first presented by Oliner *et al.*,[4] and extends the original observation of *MDM2* amplification in MFHs, liposarcomas and osteosarcomas, to include leiomyosarcomas, MPNSTs, a primary embryonal rhabdomyosarcoma and an alveolar rhabdomyosarcoma cell line (RMS). In contrast to Oliner *et al.*[4] who detected *MDM2* amplification in 36% (17/47) of their malignant sarcomas, we have detected amplification in only 12.3% of this sample of malignant tumours. These results are, however, in keeping with the results of Cordon-Cardo *et al.*[14] who detected a greater than five-fold amplification of the *MDM2* gene in only 15% (11/73) of their sarcomas, with Forus *et al.*[7] who detected *MDM2* amplification in 9% (9/98) of their sarcomas, and with the results of Ladanyi *et al.*[26] who detected *MDM2* amplification in 14% (4/28) of their high-grade osteosarcomas.

MDM2 amplification was seen in both high- and low-grade tumours, as well as in primary, recurrent and metastatic tumours (Table 2). There did not appear to be any clear correlation between the degree of amplification and the histological subtype or grade, or whether the source of the tumour specimen was a primary, recurrent or metastatic tumour. These results indicate that amplification of the *MDM2* gene may be important in the development of a significant minority of a wide variety of histological subtypes of malignant soft tissue tumour, but they provide no support for a role for *MDM2* gene amplification in the development of benign soft tissue neoplasms, or locally aggressive tumours such as fibromatoses.

Whereas only 11/39 of the tumours selected for MDM2 immunohistochemical staining possessed *MDM2* gene amplification, 21/39 of the tumours in this subgroup analyzed were immunostain positive for MDM2 over-expression. Other studies are in broad agreement with these results. Cordon-Cardo

et al.[14] identified MDM2 over-expression in 37% (76/207) of their sarcomas, with gene amplification present in only 15% (11/73) of a subset of these tumours, and Lianes *et al.*[18] demonstrated MDM2 over-expression in 26/87 human bladder tumours, but only 1/26 of these tumours demonstrated *MDM2* gene amplification. These results suggest that mechanisms other than gene amplification may be important in raising cellular levels of the MDM2 protein; for example, alterations in the stability of the MDM2 protein, or in the rate of transcription or translation or of the stability of the MDM2 mRNA.

Only 6/11 of the tumours with *MDM2* gene amplification reported by Cordon-Cardo *et al.*[14] showed positive immunohistochemical staining (>20% cells positive) for MDM2 over-expression, raising the possibility that in some sarcomas the target for gene amplification on chromosome 12q13 may not be *MDM2*. The results presented here, however, have demonstrated that *MDM2* amplification accurately predicts for MDM2 over-expression. This complete correlation is corroborated by three other studies with soft tissue tumours. Leach *et al.*[15] identified *MDM2* gene amplification and MDM2 over-expression in 8/24 MFHs and liposarcomas, Florenes *et al.*[16] demonstrated *MDM2* gene amplification and over-expression in 10/98 bone and soft tissue tumours, and Nakayama *et al.*[17] identified *MDM2* gene amplification, and over-expression by Northern analysis, in each of 11/48 soft tissue tumours. These observations, the demonstration that *MDM2* inhibits transactivation by the product of the p53 gene (a gene known to contribute to the neoplastic process in sarcomas) and the demonstration that *MDM2* is a dominantly transforming oncogene[9] provide strong evidence in favour of a role for *MDM2* amplification and over-expression in the genesis of a subgroup of soft tissue sarcomas.

We have identified over-expression of both p53 and MDM2 in two tumours, STS24 and STS102, one of which possessed *MDM2* gene amplification. The status of the p53 gene in these two tumours has not been examined. However, when similar findings have been demonstrated in other tumours,[16,18] over-expression of the MDM2 protein was accompanied by over-expression of wild-type and not mutant p53 protein. For example, Landers *et al.*[27] examined choriocarcinoma cell lines and found over-expression of both wild-type p53 and MDM2. The over-expression of MDM2 was not explained by gene amplification, elevated transcription or altered protein stability, but appeared to have resulted from increased protein translation. In addition to these observations, the level of *MDM2* transcription is known to be regulated by the expression of wild-type p53.[11] Additional data regarding the status of the p53 gene were available for 38 of the tumours examined for *MDM2* gene amplification (29 leiomyosarcomas, 4 cell lines, 1 primary rhabdomyosarcoma, 2 MFHs, 1 liposarcoma and 1 post-irradiation spindle cell tumour), and nine of these had also been examined for MDM2 over-expression. Thirteen tumours possessed p53 mutations, seven had undergone homozygous rearrangement of the p53 gene and six had mis-sense point mutations.[24,28] Two of these tumours, STS7, an MFH, and STS117, a liposarcoma, both of which had undergone homozygous rearrangement at the p53 locus, also demonstrated MDM2 over-expression. In STS117 the over-expression was found in the absence of gene amplification. The status of wild-type p53 protein expression in tumour cells may, therefore, modulate the level of MDM2 protein expression, thus providing a mechanism for MDM2 over-expression in the absence of gene amplification in a subgroup of tumour cells.

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