Amplification of the MDM2 gene in human breast cancer and its association with MDM2 and p53 protein status

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Summary

The present study reports on the frequency of MDM2 gene amplification and MDM2 protein expression in a series of 100 breast carcinomas and its association with accumulation of the p53 protein. Of the 100 cases, frozen samples for 82 cases were available for Southern blotting. Three of the 82 (4%) demonstrated MDM2 gene amplification of up to 6-fold. Immunohistochemical analysis of the formalin-fixed, paraffin-embedded tumours demonstrated that 79 (79%) had nuclear expression for MDM2 in 10–50% of the tumour cells (type 2 staining) and were denoted MDM2⁺. Two of the MDM2-amplified samples were MDM2⁺ with one of the two tumours also displaying type 2 p53 nuclear staining. Finally at the protein level, MDM2⁺ tumours were significantly associated with tumours having low levels of p53 staining (0–10%) cells positive (P = 0.03). We conclude that MDM2 gene amplification occurs at a lower frequency in breast cancer than in non-epithelial tumours. Alterations in MDM2 and p53 may represent alternative pathways in tumorigenesis, but they are not mutually exclusive in all cases.

Keywords: MDM2; breast; p53; microwave; amplification

MDM2 is an evolutionarily conserved gene (Fakhrazadeh et al., 1991) which was originally identified as a highly amplified gene present on double minutes in a spontaneously transformed tumorigenic derivative of a Balb/c cell line called 3T3DM (Cahilly-Snyder et al., 1987). Subsequently, Oliner et al. (1992) cloned the human MDM2 gene and localised it to chromosome 12q13–14. Analysis of the predicted amino acid sequence of the MDM2 protein suggests that it may be a DNA-binding protein or transcription factor (Fakhrazadeh et al., 1991; Oliner et al., 1992). In addition, it is also thought to function as a regulator of p53 function. This stems from evidence that the MDM2 protein forms oligomeric complexes with the p53 protein in vivo and in vitro (Momand et al., 1992; Oliner et al., 1992) and when experimentally overexpressed inhibits the transactivating capability of p53 (Momand et al., 1992). This inhibition is thought to result from the MDM2 protein binding directly to the acidic activation domain of p53, concealing it from the transcriptional machinery (Oliner et al., 1993). Apart from its role as a p53 regulator, MDM2 also has oncogenic properties, as evident from transfection studies in which MDM2 overexpression was found to increase the tumorigenic potential of NIH3T3 and Rat2 cells (Fakhrazadeh et al., 1991) and to overcome wild-type p53 suppression of transformed cell growth (Finlay, 1993). Therefore, its role in tumorigenesis and its detectable alteration in tumour samples is of clinical interest. At the DNA level, a number of groups have examined MDM2 amplification in non-epithelial tumours. Of these, MDM2 amplification ranging from 4- to 70-fold has been reported in 14% (4/28) of osteogenic sarcomas (Ladanay et al., 1993), 10% of primary brain tumours (15/157) (Reifengerberger et al., 1993) and up to 36% (17/47) of soft-tissue sarcomas (Oliner et al., 1992; Leach et al., 1993a), with no apparent amplification in Ewing tumours (Kovar et al., 1993) of myelodysplastic syndrome (Preudhomme et al., 1993), astrocytomas (Rubio et al., 1993) or leukaemia (Bueso-Ramos et al., 1993). With regard to the epithelial malignancies, none of the gastrointestinal tumours (Oliner et al., 1992), cervical cancers (Kessis et al., 1993) or breast cell lines (Sheikh et al., 1993) investigated showed evidence of aberrant MDM2 gene copy number. Interestingly, at the mRNA level, two studies found increased MDM2 expression with no apparent alteration in MDM2 gene copy number (Bueso-Ramos et al., 1993; Sheikh et al., 1993), suggesting that mechanisms other than gene amplification may play a role in deregulating the expression of MDM2.

Therefore, the objectives of the present study were as follows: firstly, to determine the frequency of MDM2 amplification in a series of breast carcinomas using Southern blotting, which to our knowledge had not been assessed in clinical breast tumour material; secondly, to investigate the use of microwave enhancement (Shi et al., 1991) for immunohistochemically evaluating the MDM2 protein in formalin-fixed, paraffin-embedded material using the IF2 mouse monoclonal antibody, which according to the originators does not work on paraffin-embedded material (Leach et al., 1993a); thirdly, to correlate MDM2 amplification status with MDM2 protein expression; and, finally, to determine the association of altered MDM2 at the DNA and protein levels with accumulation of p53.

Materials and methods

Tumours

Of the 100 tumours analysed in the present study, 73 were infiltrating ductal carcinomas (71 in females; two in male), 15 were infiltrating ductal with an in situ component (DCIS), three were pure DCIS, seven were infiltrating lobular and two were colloid carcinomas. Fresh breast samples were snap frozen in liquid nitrogen and stored at −70°C before DNA extraction. The corresponding paraffin-embedded tumours were formalin fixed and processed according to routine histological techniques.

Southern blotting

Amplification of the MDM2 gene was studied by Southern blotting analysis. Briefly, 10 μg of high molecular weight DNA from 82 frozen breast carcinomas were EcoRI digested, electrophoretically separated in 0.8% agarose gels and alkali blotted to Hybond N⁺ (Amersham) nylon membranes. Following hybridisation with a [α-32P]dCTP-labelled human MDM2 cDNA probe (C14-2), the membranes were exposed to intensifying screens for 2–10 days at −70°C. The blots were subsequently reprobed with the pDCC1.65 probe, which
contains nucleotides 591–2250 of the deleted in colorectal cancer (DCC) gene (Fearon et al., 1990). This probe, which detects fragments of similar size as MDM2-probed EcoRI DNA, was used as a control probe similar to a previous study (Oliner et al., 1992). Hybridisation conditions were similar to those previously used (Oliner et al., 1992). Tumour samples were considered amplified following (a) comparison of amplified signals to germline placental control, (b) evaluation of consistent well loading by examination of ethidium bromide-stained agarose gels before Southern transfer and by reprobing with the control gene probe (pDCC 1.65) and, finally, (c) serial dilutional analysis of amplified samples. The probes were kindly donated by Dr J Oliner and Dr B Vogels-tein (Johns Hopkins University, Baltimore, MD, USA).

Immunohistochemistry
The MDM2 and p53 proteins were immunohistochemically assessed on air-dried 5 μm formalin-fixed, paraffin-embedded sections using the commercially available IF2 (Oncogene Science) and NCL-p53 DO7 (Novacastra Laboratories) mouse monoclonal antibodies respectively. In the case of MDM2, immunoreactivity was not detectable on paraffin-embedded sections using IF2 and standard immunohistochemical procedures. Microwaving of the sections before application of the antibody proved successful. Briefly, the sections were placed in a microwaveable trough, submerged in 10 mm citrate buffer (pH 6.01), wrapped in vented clingfilm and incubated for two 5 min periods at maximum power in a domestic microwave (Belfing model MW 820T-800W). Following microwaving, the sections were allowed to come to room temperature in the buffer and rinsed in distilled water. The MDM2 (IF2) antibody was applied to the sections at a 1:75 dilution for 10 min at room temperature.

The p53 antibody (DO7) was used at a 1:50 dilution for 1 h at room temperature. Comparative studies of frozen sections using this protocol showed good correlation and, therefore, microwave enhancement was not investigated for this antibody. There have been reports, however, that such treatment may enhance staining with this antibody (Gown et al., 1993). Immunoreactivity for both p53 and MDM2 was demonstrated using the universal labelled streptavidin–biotin (LSAB), horseradish peroxidase (HRP) kit (Dako) according to the manufacturer’s instructions. The sections were counterstained in 0.3% methyl green and scored semiquantitatively following scanning of the entire tumour field. Nuclear immunoreactivity for MDM2 and p53 was scored as negative (0%), type 1 (<10% nuclei positive), type 2 (10–50% nuclei positive) and type 3 (>50% nuclei positive). The significance of MDM2 nuclear staining with other clinicopathological features including accumulated p53 status was assessed using the χ²-test.

Results
Amplification
In the present study, frozen tissue was available for Southern blotting analysis in 82/100 of cases. Of these, MDM2 amplification was present in 4% (3/82) (Figure 1, blots 1a and 2a), and these were histologically classified as infiltrating ductal. The corresponding blots showing reprobing with the control probe pDCC1.65 are also shown (Figure 1, blots 1b and 2b respectively). Sample 10 (Figure 1, blot 1a), demonstrated the highest gene copy number based on serial dilutional analysis (4- to 6-fold) and was from a patient whose mother died from breast cancer at the age of 48 and whose brother died at 25 from liver cancer with an unknown

Figure 1 Southern blot analysis of MDM2 gene amplification in EcoRI restricted breast DNA samples hybridised with a human MDM2 cDNA fragment (C-142) (blots 1a and 2a) with corresponding reprobing of the blots with pDCC1.65 (blots 1b and 2b respectively). The case numbers are given on the tops of each lane. Tumour DNA samples 10, 16 and 19 show amplification for MDM2 (see arrows). Germine placental controls are indicated (P) and approximate DNA fragment sizes are shown on the left in kilobases (kb).
primary. The remaining two MDM2-amplified samples (tumours 16 and 19) (Figure 1, blot 2a) had a 2-4 fold amplification based on serial dilutional analysis.

Expression

Immunoreactivity for the MDM2 and p53 gene products was predominantly located in the nucleus (Figure 2a and b respectively), with some weak cytoplasmic staining. Of the 97 samples assayed, 7% (7/97) had type 2 MDM2 nuclear staining (10-50% of tumour nuclei positive (MDM2+) (Table I). With the exception of one case of ductal carcinoma in situ (DCIS) (histologically typed as cribriform), all the MDM2+ cases were infiltrating ductal carcinomas. None of the tumours had type 3 staining (>50% nuclei positive). Type 1 and negative staining frequencies are detailed in Table I, as are the frequencies for p53 nuclear accumulation.

Comparing MDM2 amplification with MDM2 and p53 nuclear protein expression, 2/3 MDM2-amplified samples were MDM2+ (type 2 staining) and one was negative (Table II). Of the three amplified samples, only one had gross accumulation of p53 (type 2 staining; sample 16). The two remaining amplified tumours consisted of one tumour negative for p53 (sample 19) and one with type 1 staining (<10% nuclear positivity; sample 10) (Table II). Finally, 95 cases were assayed for both p53 and MDM2 protein expression using immunohistochemistry. Chi-square analysis indicated that MDM2+ status was significantly associated (P = 0.03) with low levels of p53 (negative and type 1 staining; Table III), with 6/7 MDM2+ tumours having this p53 profile. Of note is the fact that 5/7 of these MDM2+ tumours had no underlying gene amplification (Table II). With regard to the other clinicopathological variables looked at, MDM2+ status was not associated with age, tumour grade, lymph node status or tumour size (data not shown).

Discussion

In the present study, we investigated the frequency of MDM2 alteration at both the DNA and protein levels in an epithelial tumour, namely breast cancer, and correlated the findings with immunohistochemical accumulated p53 status. Our investigation was prompted by the fact that other studies on epithelial tumours or cell lines (Oliner et al., 1992; Kessis et al., 1993; Sheikh et al., 1993) found no MDM2 gene amplification. In the present study, altered MDM2 gene copy number was evident in 4% of breast carcinomas. This frequency is lower than that reported in the literature for non-epithelial malignancies (Oliner et al., 1992; Ladanyi et al., 1993; Reifenberger et al., 1993; Leach et al., 1993a), and is one of the first reports of MDM2 amplification in epithelial tumours. The clinical significance of such amplification is yet to be clarified. In the study of Ladanyi et al. (1993), increased MDM2 gene copy number was detected more frequently in metastatic or recurrent rather than in primary high-grade osteosarcomas (P = 0.02) suggesting that such alteration may be associated with tumour progression. The finding of a 2-fold increase in MDM2 copy number and RNA expression in a recurrent glioblastoma sample compared with the primary lesion supports this view (Reifenberger et al., 1993).

In the present study, the highest degree of amplification was present in tumour DNA from a woman who appeared to have a family history of cancer, suggesting that investigations of MDM2 alterations in cancer families may be of interest.

Table I Frequency of MDM2 and p53 nuclear staining

| Antigen | Type 3 (>50%) | Type 2 (10-50%) | Type 1 (<10%) | Negative (-) |
|---------|---------------|-----------------|---------------|--------------|
| MDM (n = 97) | - | 7 (7%) | 14 (15%) | 76 (78%) |
| p53 (n = 97) | 28 (29%) | 10 (10%) | 19 (20%) | 40 (41%) |

*Percentage of tumour nuclei with immunoreactivity.

Table II Association of altered MDM2 with accumulated p53 status

| Samples | MDM2 copy number | MDM2 expression | Accumulated p53 status |
|---------|------------------|-----------------|------------------------|
| Amplified | | | |
| 10 | 4-6 | + | Type 1 |
| 16 | 2-4 | + | Type 2 |
| 19 | 2-4 | Negative | Negative |
| Non-amplified | | | |
| 15 | - | + | Type 1 |
| 30 | - | + | Negative |
| 45 | - | + | Negative |
| 47 | - | + | Type 1 |
| 60 | - | + | Type 1 |

*+ indicates type 2 MDM2 staining (10-50% of tumour nuclei positive). p53 nuclear accumulation as described in the Materials and methods section.

Table III Association of MDM2 protein status with nuclear p53 staining

| p53 nuclear accumulation | Total no. of cases | Type 1 (10-50%) | Type 1 (<10%) | Negative |
|--------------------------|--------------------|-----------------|---------------|---------|
| Type 2 and 3 | 37 | 1 | 2 | 34 |
| Type 1 and negative | 58 | 6 | 12 | 40 |
| Total | 95 | 7 | 14 | 74 |

*p53 nuclear accumulation as described in the Materials and methods section. *MDM2 nuclear expression as described in Materials and methods section. Significance level P = 0.03 using chi² analysis.
This stems from reports of a lack of p53 mutations in exons 5–9 of familial breast cancer patients (Prosser et al., 1991; Warren et al., 1992), suggesting p53 mutations may not contribute to hereditary breast cancer. Is it possible that MDM2 could play a role in these cases? It is hard to conceive however, how gene amplification could be inherited, and how this may manifest other than predisposing to an amplification event. One possibility is that an inherited mutation in a gene responsible for the fidelity of DNA replication, such as MSH2 (Leach et al., 1993b), could result in genomic instability leading to gene amplification.

Microwave-based antigenic unmasking has recently been evaluated by our group (Kelleher et al., 1994) and others, and is a recommended technique for use in paraffin material for detecting a wide range of antigens (Shi et al., 1991; Gown et al., 1993). The frequency of 7% positive immunostaining for the MDM2 protein is slightly higher than the altered frequency we report at the DNA level and may suggest that mechanisms other than gene amplification can lead to aberrant MDM2 expression, for example oestrogen may modulate MDM2 mRNA expression (Shiekh et al., 1993).

The fact that one of our amplified samples had no apparent alteration in MDM2 protein expression suggests that in a subset of tumours the amplification event could be driven by a different gene within this chromosomal region. Possible candidate genes are CDK4 or gli, which have been reported to be co-amplified with MDM2 in an osteosarcoma cell line (Khatib et al., 1993). The 12q13–14 amplicon may therefore represent a similar situation to that found with the 11q13 regional locus in breast carcinomas, in which int-2, although not expressed, is frequently co-amplified with hat-1 and bcl-1 (cyclind/PRA/D1), with the latter reported to have elevated transcription accompanied by its amplification (Yoshida et al., 1993).

Looking at MDM2 gene amplification in association with accumulated p53 status, 1/3 amplified MDM2+ tumours demonstrated type 2 p53 nuclear staining (10–50% of tumour nuclei positive). This is similar to a previous study (Reifenberger et al., 1993) in which two MDM2-amplified samples displayed significantly increased levels of p53 mRNA, with one case showing concomitant p53 nuclear expression in the majority of the tumour nuclei as determined using the DO7 antibody. The significance of this accumulation in the present study is difficult to interpret without sequence analysis. Firstly, accumulation of p53 in the majority of cases has been shown to be synonymous with the presence of underlying p53 mutations (Singh et al., 1993). These mutations, however, have not been found in any of the MDM2-amplified samples reported in the literature (Oliner et al., 1992; Reifenberger et al., 1993; Leach et al., 1993a), suggesting that MDM2 amplification and p53 exon mutations may be mutually exclusive. Secondly, based on the study of Barnes et al. (1992) reporting on the accumulation of non-mutated, wild type p53 in a familial case, we used the DO7 antibody in our study as it detects both the wild-type and mutant p53 forms. Therefore, the accumulation of p53 in this amplified tumour could be of either phenotype and may be due to sequestering by MDM2. Of course, other mechanisms could lead to p53 accumulation resulting from intrinsic mutations, altered splice patterns, increased protein stability or sequestering by other host proteins. The prognostic role of MDM2 amplification in breast carcinomas (Oliner et al., 1993) and the identification of multiple MDM2 proteins and possible MDM2–p53 protein complexes (Olson et al., 1993) further underlines the complexity of maintaining p53 equilibrium.

In conclusion, our study reports on a frequency of 4% for amplification of the MDM2 gene in human breast cancer and expression of the MDM2 gene product (10–50% nuclei positive) in 7% of samples, and is one of the first reports of such an alteration in epithelial tumours. Based on this frequency, it is unlikely that altered MDM2 will have significant clinical value, but this remains to be determined. The fact that 6/7 MDM2-positive protein samples were p53 negative (P = 0.03) indicates that alterations in MDM2 and p53 may represent alternative pathways in tumorigenesis. Finally, the role of MDM2 alteration in familial/inherited dispositions may be warranted.

**Abbreviations**

MDM2, murine double minute 2; cDNA, complementary DNA; DCIS, ductal carcinoma in situ; ER, oestrogen receptor.

**Acknowledgements**

The authors wish to thank Professor William Gullick for helpful discussions pertaining to this work and for critical review of the manuscript. Special thanks to the photographic department of the Audio Visual Centre, University College Dublin. This study was funded in part by the Health Research Board (HRB) Ireland.

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