EXPRESSION OF MDM2 DURING MAMMARY TUMORIGENESIS

Jan Pinkas[1,2], Stephen P. Naber[3], Janet S. Butel[4], Daniel Medina[5] and D. Joseph Jerry[1,2]*

[1]*Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, MA, USA*
[2]*Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA, USA*
[3]*Department of Pathology, Baystate Medical Center, Springfield, MA, USA*
[4]*Division of Molecular Virology, Baylor College of Medicine, Houston, TX, USA*
[5]*Department of Cell Biology, Baylor College of Medicine, Houston, TX, USA*

The MDM2 oncoprotein encodes a 90 kDa nuclear phosphoprotein capable of abrogating the growth suppressive functions of p53 and pRb tumor suppressor proteins by direct interaction. Alternative splicing of MDM2 protein coding sequences has been documented during tumor progression in human ovarian and bladder carcinomas. The aim of this study was to determine whether alternative splicing of MDM2 occurs during breast tumorigenesis in mice and humans and whether protein coding sequences were affected. Specimens representing normal and malignant breast tissues from the murine D2 mammary tumor model system and human breast carcinomas were examined. Three distinct mdm2 mRNA transcripts of 3.3, 1.6 and 1.5 kb were detected in normal and malignant murine mammary tissues by Northern blot analysis using a full-length *mdm2* cDNA probe. Additional Northern blot analysis using a probe derived from exon 12 of murine *mdm2* demonstrated that the 1.5 and 1.6 kb transcripts lack sequences encoding the C-terminus of the protein. No evidence of internal deletions of protein coding sequences of mdm2 was detected in any of the normal mammary tissues or D2 murine mammary tumors examined by reverse transcription PCR (RT-PCR). Three distinct MDM2 transcripts of 6.7, 4.7 and 1.9 kb were detected in malignant human breast tissue by Northern blot analysis using a cDNA probe specific for the complete open reading frame of human *MDM2*. However, a cDNA probe specific for the last exon of human *MDM2* hybridized only to the 6.7 and 4.7 kb transcripts, demonstrating that the 1.9 kb transcript lacked protein coding sequences contained in exon 12. Similarly, no internal deletions were detected in a panel of malignant human breast tissues using RT-PCR and analogous primers within human *MDM2*. Therefore, breast tumors differ from other solid tumors reported previously in that no internal deletions of MDM2 protein coding sequences were observed. However, the data document the presence of multiple MDM2 mRNA transcripts in both normal and malignant breast tissues. A subset of MDM2 transcripts were shown to lack the last exon which contains sequences coding for the RING and zinc fingers and domains which are targets for caspase-3 mediated proteolytic degradation and are required to target p53 for proteosomal degradation. *Int. J. Cancer* 81:292–298, 1999.
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The murine double minute 2 (*mdm2*) proto-oncogene was originally cloned from a transformed mouse NIH 3T3 cell line where it was amplified more than 50-fold (Fakharzadeh *et al.,* 1991). Cell lines selected for amplification of *mdm2* were tumorigenic upon subcutaneous injection into athymic (nude) mice (Fakharzadeh *et al.,* 1991). Subsequent analyses demonstrated that MDM2 can interact with and inhibit transcriptional activation by the p53 tumor suppressor protein (Momand *et al.,* 1992). The direct interaction of MDM2 with the p53 protein is believed to mediate its oncogenic effects. However, even when MDM2 protein is expressed at very high levels within cells, a relatively small proportion of the p53 protein is found complexed with the MDM2 protein (Barak *et al.,* 1994; Momand and Zambetti, 1996). In addition, alternate forms of MDM2 mRNA that lack internal coding sequences were discovered in rodent cell lines (Fakharzadeh *et al.,* 1991; Barak *et al.,* 1994) and more recently, in primary bladder and ovarian carcinomas in humans (Sigalas et al., 1996). The cellular

roles of these truncated MDM2 isoforms found in human and rodent cell lines and tissues remains to be determined.
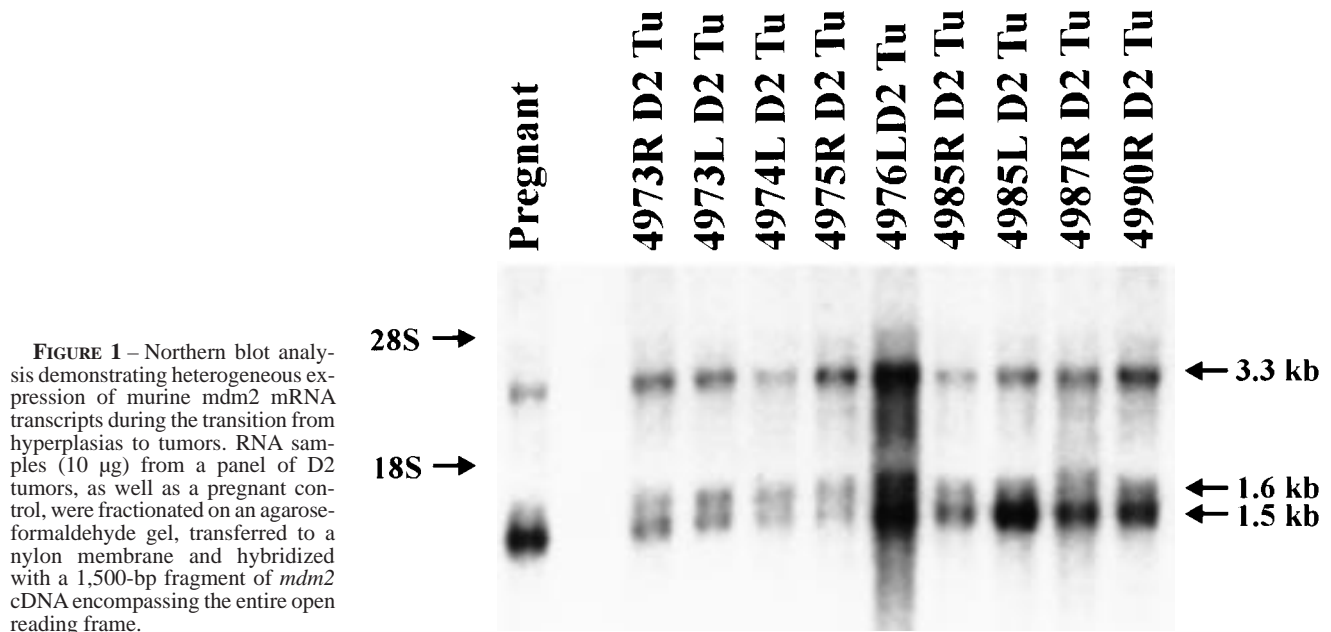
The structure of the MDM2 protein suggests that it may have roles in addition to binding p53. The MDM2 protein contains a zinc finger, an acidic domain, a nuclear localization signal typical of DNA-binding proteins and a RING finger motif, which may mediate interactions with other proteins and RNA, in addition to the well-characterized p53-binding domain (Piette *et al.,* 1997). The possibility that MDM2 may act in a p53-independent fashion was demonstrated by its ability to bind and activate E2F1/DP1 transcription factor and inhibit pRb-mediated cell cycle arrest (Piette *et al.,* 1997). The E2F1-binding domain of MDM2 has been mapped to the N-terminal 220 amino acids and is thought to overlap with the p53-binding domain (Piette *et al.,* 1997). The N-terminal 134 amino acids of MDM2 transformed a p53-/pRb-osteosarcoma cell line through inhibition of G1 cell cycle arrest mediated by p107, a member of the pRb family (Dubs-Poterszman *et al.,* 1995). These results demonstrate that the N-terminus of MDM2 interacts with the pRb family of tumor suppressors, as well as p53, resulting in the inhibition of their cell cycle arrest activities.

Indeed, MDM2 appears to act through p53-dependent and -independent mechanisms of tumorigenesis in human tumors. Amplification of the *MDM2* gene was seen in 20–35% of benign and malignant sarcomas (Momand *et al.,* 1998). An inverse association between amplification of *MDM2* and mutation of p53 was found in sarcomas (Momand *et al.,* 1998), suggesting that these are redundant targets, both of which lead to inactivation of the p53 pathway. In contrast, *MDM2* was amplified in 5% of breast carcinomas (McCann *et al.,* 1995), and no consistent correlation was seen between overexpression of MDM2 and wild-type p53 (Bueso-Ramos *et al.,* 1996). Analysis of a portion of the protein coding sequence in MDM2 mRNA from human breast carcinomas failed to detect alternatively spliced transcripts (Bueso-Ramos *et al.,* 1996). However, Western blot analysis demonstrated that multiple truncated isoforms of 85, 76/74 and 57 kDa, in addition to the 90 kDa full-length protein, were expressed in this panel of breast carcinomas (Bueso-Ramos *et al.,* 1996). Evidence from human bronchogenic carcinomas indicated that the 76/74 kDa MDM2 isoforms lacked N-terminal sequences contained within the p53-binding domain, and that the 57 kDa isoform lacked the C-terminal RING and zinc finger domains (Gorgoulis *et al.,* 1996). Analysis of the complete open reading frame of MDM2 mRNA transcripts in bladder and ovarian carcinomas revealed alternative splicing of internal protein coding sequences in 50% of the tumors

FIGURE 1 – Northern blot analysis demonstrating heterogeneous expression of murine mdm2 mRNA transcripts during the transition from hyperplasias to tumors. RNA samples (10 μg) from a panel of D2 tumors, as well as a pregnant control, were fractionated on an agarose-formaldehyde gel, transferred to a nylon membrane and hybridized with a 1,500-bp fragment of *mdm2* cDNA encompassing the entire open reading frame.

examined (Sigalas *et al.,* 1996). The truncated MDM2 transcripts isolated from these tumors coded for protein isoforms that were more highly transforming than the full-length protein (Sigalas *et al.,* 1996). This suggested that alternatively spliced MDM2 mRNA transcripts lacking protein coding sequences are expressed in malignant human breast tissues.

A potential role for *mdm2* in breast tumorigenesis in the mouse has been described. Targeted expression of mdm2 to the mammary gland in transgenic mice inhibited normal mammary gland development and resulted in the development of mammary tumors (Lundgren *et al.,* 1997). Interestingly, the phenotype of the *mdm2* transgenic mice was similar in the $p53^{+/+}$ and $p53^{-/-}$ background suggesting that *mdm2* can mediate transformation through p53-independent mechanisms (Lundgren *et al.,* 1997). However, the role of mdm2 in a spontaneous model of breast tumorigenesis in the mouse has not been examined. Therefore, experiments were undertaken to determine whether alternative splicing of MDM2 protein coding sequences occurs during breast tumorigenesis using the D2 murine mammary tumor model system and malignant human breast tissues. RT-PCR analyses performed on a panel of D2 murine mammary tumors and malignant human breast tissues failed to detect any deletions of N-terminal protein coding sequences. Multiple MDM2 mRNA transcripts were detected by Northern blot analysis and were shown to lack protein coding sequences for the RING and zinc fingers contained in exon 12.

MATERIAL AND METHODS

Tissues

Mammary tumors were derived from *in vivo* outgrowths of D2 preneoplasias. The D2 line was established from hyperplastic foci in BALB/c mice that were stimulated hormonally by pituitary isografts (Medina., 1996). Mice bearing D2 preneoplastic outgrowths developed a moderate incidence of mammary tumors within 12 months after transplantation (approximately 60%). Normal mammary tissues were obtained from the 4th inguinal glands of pregnant 15 day female BALB/c mice (8 to 12 week-old). Human breast tissues were obtained from the frozen tissue bank at Baystate Medical Center (Springfield, MA). The human breast samples consisted of 19 infiltrating ductal carcinomas (grades I–III).

Blot hybridization

RNA was isolated from tissues using Ultraspec (Cinna/Biotecx, Houston, TX). Total RNA was fractionated on 1.25% agarose gels in a MOPS/formaldehyde buffer (40 mM MOPS, 10 mM NaOAc, 1 mM EDTA, and 1 M formaldehyde), then transferred to Zetabind (Cuno, Meriden, CT) and crosslinked with 254 nm uv light. Equal loadings were assured by UV shadowing. DNA probes were labeled with ^{32}P-dCTP by random priming, then hybridized to the filters. *MDM2* cDNA probes for blot hybridization were generated by PCR using sense primers (5′-TGAAGGGTCGGAAGAT-GCG-3′ and 5′-AAGATGCTGGACCCCTTAGTG-3′) with an anti-sense primer (5′-GTGAGCAGGTCAGCTAGTTG-3′) to amplify the 1,500-bp full-length and 529-bp exon 12 murine *mdm2* probes, respectively. Sense primers (5′- GAGGAGCAGGCAAATGTGC-3′ and 5′-CCTTCGTGAGAATTGGCTTC-3′) with an antisense primer (5′-CATACTGGGCAGGGCTTATTC-3′) were used to amplify the 1,445-bp full-length and 461-bp exon 12 human *MDM2* probes, respectively.

RT-PCR

Mammary tumors were carefully dissected to remove stromal tissue. Total RNA (1 μg) was reverse transcribed at 42°C by using random hexamers to prime the first strand cDNA synthesis reaction. Transcripts of MDM2 were amplified using the primer pairs described below. The primers were designed to distinguish transcripts that encode the full-length N-terminal region of MDM2 from isoforms bearing N-terminal deletions. The primers were synthesized by AMITOF (Allston, MA). The sense and antisense primers were designated by the prefixes 5′- and 3′-, respectively. The murine mdm2 primers used were 5′A (5′- CCAGGCCAATGT-GCAATACC-3′), 5′D (5′-AAGATGCTGGACCCCTTAGTG-3′), 3′D (5′- CTCGGATCAAAGGACAGGGAC-3′) and 3′MID (5′-TCCTCCTCAGCACATGGCTC-3′). The human *MDM2* primers used were H5′A (5′-GAGGAGCAGGCAAATGTGC-3′), H5′D (5′-CCTTCGTGAGAATTGGCTTC-3′) and H3′MID (5′-TCCT-CAACACATGACTCTCTGG- 3′). AMV-RTase (Seikagaku, Rockville, MD) was used for cDNA synthesis. Reagents for PCR were purchased from Perkin-Elmer/Cetus (Norwalk, CT). The conditions for amplification were denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 52°C for 1 min and 72°C for 2 min. A 10 μl aliquot of each 50 μl reaction volume was

analyzed by agarose gel electrophoresis and visualized by staining with ethidium bromide.

RESULTS

Multiple mdm2 mRNA transcripts are expressed in mouse mammary tissues

Expression of mdm2 mRNA was compared in murine D2 mammary tumors and in pregnant mammary tissue to ascertain whether multiple mRNA species were expressed. Three major transcripts (3.3, 1.6 and 1.5 kb) were detected in normal and malignant murine mammary tissues (Fig. 1). The 3.3 kb transcript corresponds to the full-length mRNA identified during the initial cloning of *mdm2* (Fakharzadeh *et al.,* 1991). The nucleotide sequence of the 1.6 and 1.5 kb transcripts is unknown, but both alternate transcripts are potentially large enough to encode the full-length mdm2 protein. No novel mRNA transcripts were expressed at detectable levels in the D2 tumors as compared with the pregnant mammary tissue. The relative levels of all 3 transcripts varied between normal mammary tissue and the D2 tumors, but no consistent difference in the pattern or levels of mdm2 mRNA transcripts was evident.

To determine whether the truncated mdm2 transcripts contained deletions of coding sequences, exon-specific probes were used in Northern blot analyses of a panel of D2 tumors and pregnant

mammary tissue. A cDNA probe corresponding to the complete open reading frame of murine *mdm2* hybridized to 3 transcripts as seen earlier (Figs. 1, 2*a*), and small probes derived from 5′ sequences (exons 1–9) also hybridized to all three mdm2 mRNA transcripts (data not shown). However, an *mdm2* cDNA probe derived from exon 12 only hybridized to the 3.3 kb transcript (Fig. 2*b*). This suggested that the 1.6 and 1.5 kb mdm2 mRNA transcripts lacked exon 12, but maintained 5′ coding sequences.

RT-PCR analysis of mdm2 in murine breast tissues

Primers were designed to amplify the complete *mdm2* open reading frame to determine whether the alternate transcripts seen by Northern blot (Figs. 1, 2) were generated by internal deletion of coding sequences. The murine *mdm2* gene contains 12 exons with exons 1 and 2 being non-coding (Fig. 3) (Jones *et al.,* 1996). Sense primer 5′A (complementary to sequences in exon 3) was used in conjunction with antisense primers 3′MID (complementary to sequences in the middle of exon 12) and 3′D (complementary to sequences in exon 9) (Jones *et al.,* 1996). Amplification of mdm2 mRNA from D2 tumors and the pregnant control with the 5′A-3′MID primer pair yielded a 1,134-bp PCR product expected from full-length 3.3 kb mdm2 transcripts (Fig. 4*a*). Additional RT-PCR analyses using the 3′D primer, which is contained in all 3 mdm2 transcripts, resulted in the amplification of a single 573-bp fragment in all tissues examined (Fig. 4*b*). No evidence of internal
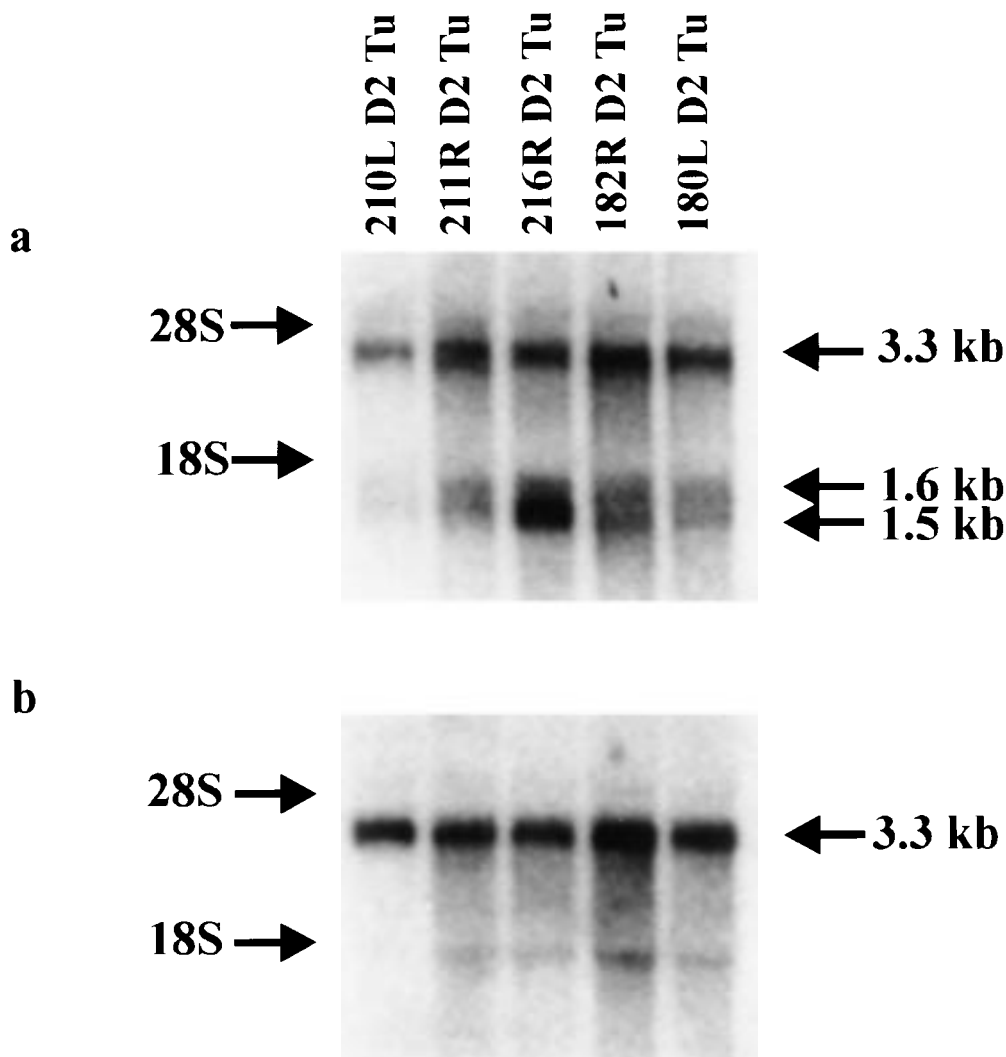

FIGURE 2 – Northern blot analysis of murine mdm2 mRNA transcripts expressed in mammary tumors derived from D2 preneoplastic outgrowths. RNA samples (10 μg) from a panel of D2 tumors were fractionated on an agarose-formaldehyde gel, transferred to a nylon membrane and hybridized with (*a*) a 1,500-bp cDNA probe corresponding to the complete open reading frame of murine *mdm2* and (*b*) a 529-bp cDNA probe from exon 12 of murine *mdm2*.
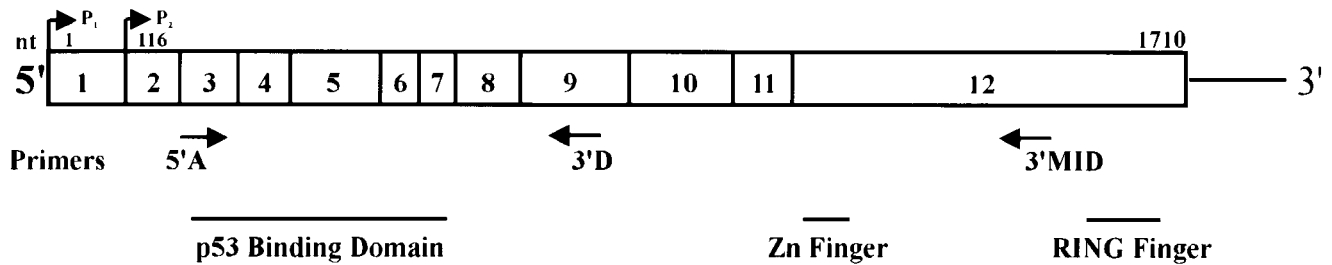
FIGURE 3 – Schematic representation of the murine *mdm2* gene with locations of the primers for RT-PCR and functional domains. P1, transcriptional start-site for the p53-independent promoter; P2, transcriptional start-site for the p53-dependent promoter; nt, nucleotide number. All locations are based on the nucleotide sequence for murine *mdm2* (Fakharzadeh *et al.,* 1991).
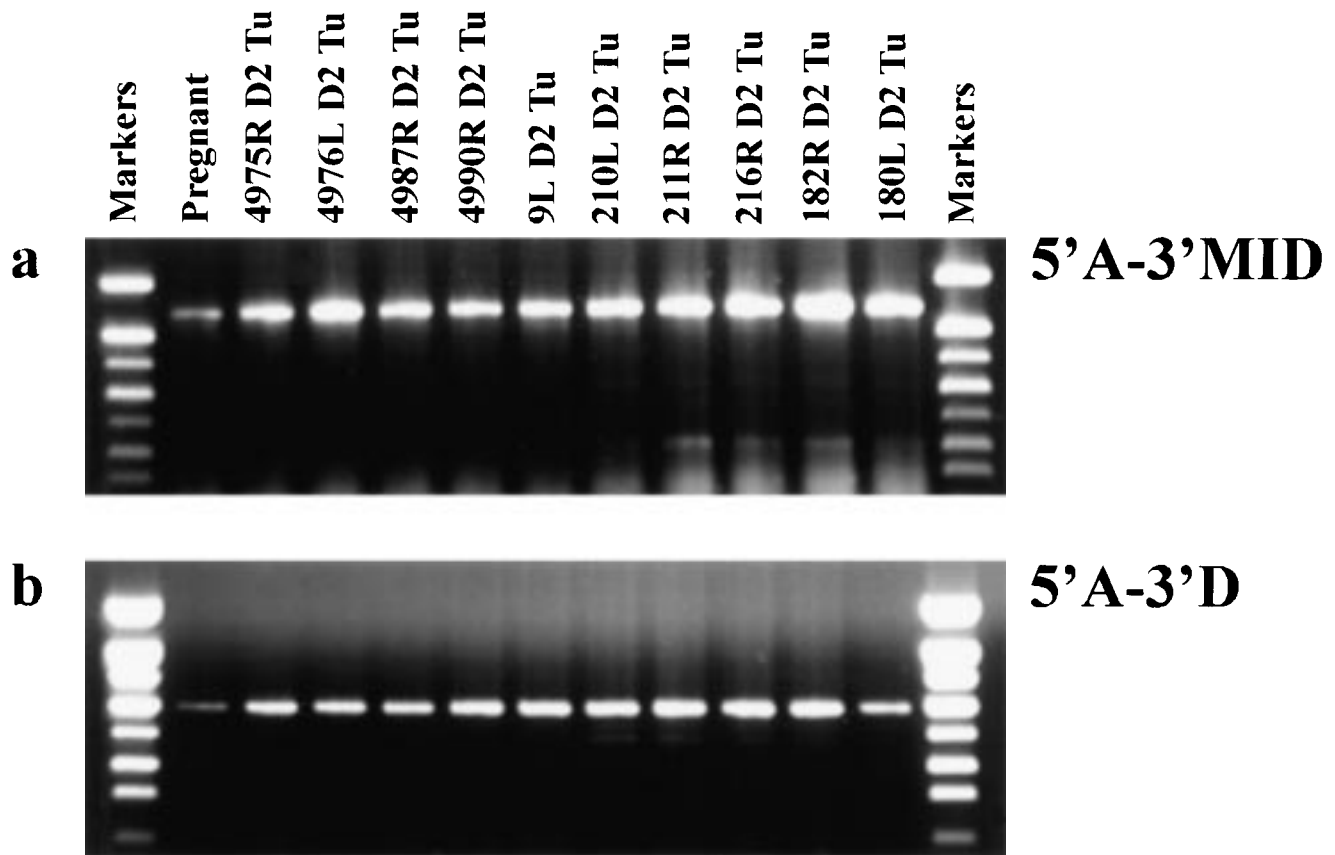

FIGURE 4 – RT-PCR analysis of murine mdm2 mRNA transcripts expressed in pregnant mammary tissue and D2 tumors. Total RNA (1 µg) was amplified by RT-PCR using (*a*) the 5′A-3′MID and (*b*) the 5′A-3′D primer pairs. The products were separated on a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination. Sizes of m.w. markers are 1444, 943, 754, 585, 458, 341, 258 and 153 bp.

deletions of coding sequences were seen in any of the mdm2 mRNA transcripts as determined by RT-PCR in 15 D2 tumors.

Analysis of the truncated human MDM2 mRNA transcripts

Northern blot analysis of MDM2 mRNA expression in human infiltrating ductal carcinomas was undertaken using analogous full-length and exon-specific human *MDM2* cDNA probes. Hybridization with a full-length human *MDM2* cDNA probe identified 3 distinct mRNA transcripts (Fig. 5*a*). The sizes of the 3 mRNA transcripts were determined to be 6.7, 4.7 and 1.9 kb after electrophoresis of poly(A$^+$) mRNA with RNA m.w. standards (data not shown). The 6.7 and 4.7 kb mRNA transcripts correspond to MDM2 mRNAs identified previously in human breast carcinoma cell lines, whereas the 1.9 kb transcript was not observed in these

cell lines (Gudas *et al.,* 1995). The levels of MDM2 mRNA varied quite markedly with only one breast carcinoma (IDC18) expressing high levels of the 6.7 and 4.7 kb transcripts. A cDNA probe specific for the last exon of human *MDM2* hybridized only to the 6.7 and 4.7 kb transcripts (Fig. 5*b*), which is consistent with exon 12 being absent from the 1.9 kb transcript. Some residual non-specific hybridization to the 18S ribosomal RNA was seen because the 1.9 kb transcript co-migrates with the 18S rRNA. To confirm this result, the levels of all 3 MDM2 transcripts were quantitated by phosphorimage analysis. This demonstrated that the ratios of the 6.7 and 4.7 kb mRNA transcripts were the same with the full-length and exon 12-specific probes but that the levels of the 1.9 kb transcript were reduced >80% in the blot probed with the exon
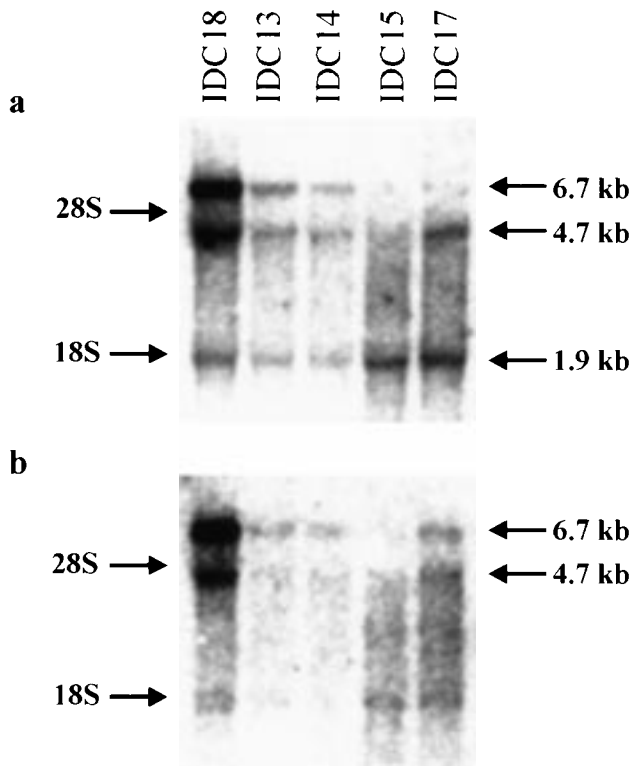
FIGURE 5 – Northern blot analysis of truncated human MDM2 mRNA transcripts demonstrating variable levels of expression in invasive ductal carcinomas. RNA samples (10 μg) from a panel of invasive ductal carcinomas (IDC13–15, 17 and 18) were fractionated on an agarose- formaldehyde gel, transferred to a nylon membrane and hybridized with (*a*) a 1,445-bp cDNA probe corresponding to the complete open reading frame and (*b*) a 461-bp cDNA probe from exon 12 of human *MDM2*.
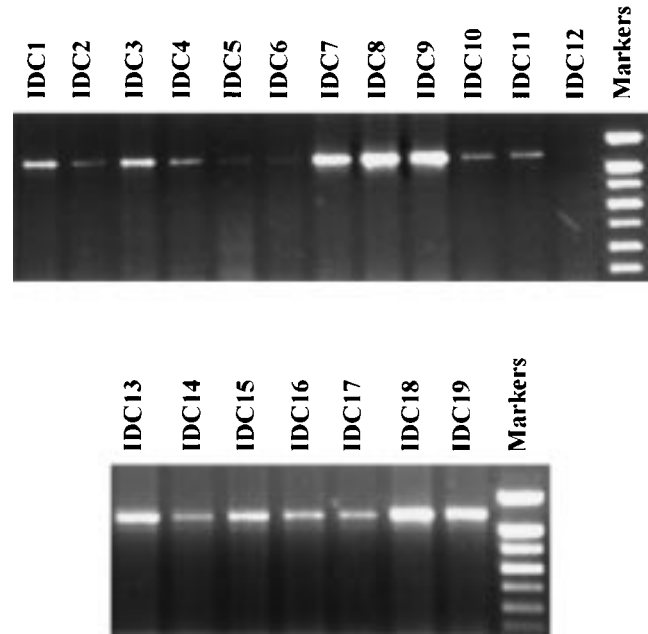

FIGURE 6 – RT-PCR analysis of human MDM2 mRNA transcripts expressed in malignant breast tissue. Total RNA (1 μg) isolated from infiltrating ductal carcinomas (IDC1–19) was amplified by RT-PCR using the H5′A- H3′MID primer pair. The products were separated on a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination. Sizes of m.w. markers are 1444, 943, 754, 585, 458, 341, 258 and 153 bp.

12-specific probe as compared with the full-length *MDM2*cDNA probe.

RT-PCR analysis of MDM2 in human breast tissues

To correlate the findings in murine mammary tumors with human breast tumors, a panel of infiltrating ductal carcinomas was examined by RT-PCR for alternative splicing of internal MDM2 protein coding sequences. The complete exon structure of human the *MDM2* gene has not been reported, but the murine and human homologs share greater then 80% identity at the amino acid level and greater then 90% identity at the nucleotide level. The genomic sequence of the first 3 exons of human *MDM2* have been reported and show conservation of exon structure and promoter sequences with murine *mdm2* (Zauberman *et al.,* 1995). Specific primers for human *MDM2* that are homologous to the murine primers described earlier were used to analyze the structure of MDM2 mRNA transcripts expressed in human breast tissues. The H5′A-H3′MID primer pair amplified a 1,142-bp PCR product in 18 of 19 of the human invasive ductal carcinomas (Fig. 6, IDC1-IDC19). No evidence of truncated MDM2 transcripts containing deletions of internal coding sequences were seen in any of the malignant breast tissues.

DISCUSSION

Aberrant patterns of expression of wild-type p53 protein have been reported suggesting that the *p53* pathway can be abrogated through mechanisms other than direct mutation. Nuclear accumulation of wild-type p53 protein was observed in 36% of human breast tumors (Allred *et al.,* 1993) and 38% of murine mammary tumors arising from D2 preneoplastic outgrowths (Jerry *et al.,* 1994). Cytoplasmic sequestration of wild-type p53 protein has been observed frequently in human breast tumors as well (Moll *et al.,* 1992). However, the molecular lesions that allow proliferation of tumors despite elevated levels of wild-type p53 protein remain to be identified.

The *MDM2* proto-oncogene is an attractive candidate to mediate inactivation of p53 during tumorigenesis. It was originally suggested that MDM2 and p53 act in an autoregulatory feedback loop to control p53 transcriptional activity (Barak *et al.,* 1994). In support of this hypothesis are reports demonstrating that amplification and overexpression of MDM2 correlate strongly with wild-type p53 in a variety of human tumors. This suggested that MDM2 mediated tumorigenesis through direct interaction with and inactivation of p53. Complicating the situation are reports demonstrating that multiple MDM2 mRNA transcripts and protein isoforms are expressed in a variety of human and rodent cell lines, tissues and tumors. Alternative splicing of internal MDM2 mRNA sequences was reported in nearly 50% of human ovarian and bladder carcinomas and was strongly correlated with the more aggressive tumors (Sigalas *et al.,* 1996). However, it was not determined whether the truncated MDM2 products amplified by RT-PCR corresponded to truncated MDM2 mRNAs and protein isoforms expressed at detectable levels in that panel of tumors. Several truncated MDM2 isoforms of 76/74 and 57 kDa were identified in a panel of human breast carcinomas by Western blot analysis, suggesting alternative splicing may occur, but an RT-PCR based approach, which only amplified a portion of the open reading frame, did not detect any deletion of sequences coding for the p53-binding domain (Bueso-Ramos *et al.,* 1996). Multiple MDM2 mRNA transcripts were documented in normal and malignant

human breast epithelial cell lines, but the transcripts were not analyzed for deletions of protein coding sequences (Gudas *et al.,* 1995).

In the present study, multiple MDM2 mRNA transcripts were detected in murine and human breast tissues, and a subset of the transcripts were shown to contain novel deletions of 3′ coding sequences. The relative abundance of the full-length 3.3 kb and the 1.6 and 1.5 kb alternate mRNA transcripts varied among pregnant mouse mammary tissue and D2 tumors, but no consistent differences were seen in the patterns or levels of the transcripts expressed. In human breast tumors, MDM2 mRNA transcripts of 6.7, 4.7 and 1.9 kb were detected which correlates well with MDM2 mRNAs identified in human mammary epithelial cell lines (Gudas *et al.,* 1995), but the 1.9 kb transcript has not been reported previously.

The structures of the MDM2 mRNA transcripts expressed in murine and human breast tissues were analyzed by RT-PCR using primers designed to amplify the complete coding sequences (primer pairs 5′A/3′MID and H5′A/H3′MID, Figs. 4, 6). Initially, the 5′ coding sequences of MDM2 were analyzed because previous reports have identified mRNA transcripts that resulted from differential promoter usage and alternative splicing of sequences coding for the p53-binding domain in cell lines (Fakharzadeh *et al.,* 1991; Barak *et al.,* 1994). No alternative splicing was observed in murine breast tissues by RT-PCR using 5′ primers in exons 1, 2 and 3 in conjunction with primer 3′MID and 3′D (Fig. 4 and data not shown). However, Northern blot analysis revealed that the 1.6 and 1.5 kb transcripts in mouse and the 1.9 kb transcript in human breast tissues failed to hybridize to a probe containing sequences within exon 12 (Figs. 2*b*, 5*b*). The presence of the 1.9 kb MDM2 mRNA transcript in human breast carcinomas is consistent with earlier reports demonstrating that the 57 kDa MDM2 protein isoform was not recognized by an antibody raised against an epitope in the C-terminus of the full-length protein (Gorgoulis *et al.,* 1996).

The expression of truncated MDM2 isoforms has significant functional implications with respect to p53. The full-length 90 kDa MDM2 isoform was shown to have ubiquitin ligase activity (Honda *et al.,* 1997) and to target p53 for ubiquitin-mediated proteolytic degradation (Haupt *et al.,* 1997). However, a truncated

MDM2 isoform lacking C-terminal sequences maintained its ability to interact with p53 but was unable to target it for proteolytic degradation. Complicating the situation are reports demonstrating that MDM2 is itself a target for proteolytic cleavage by caspase-3 generating a C-terminally truncated protein isoform (Erhardt *et al.,* 1997). The stability and function of the caspase-3 cleaved MDM2 protein species has not been determined. From these data, it would appear that the 57 kDa MDM2 isoform expressed in human breast tissues should be resistant to proteolytic degradation by caspase-3 while retaining the ability to interact with p53. This may represent a mechanism to sequester p53 in an inactive state but not target it for ubiquitin-mediated proteolytic degradation.

Our results clearly demonstrate that alternatively spliced forms of MDM2 mRNA lacking internal coding sequences are not expressed at detectable levels during breast tumorigenesis. In contrast, novel MDM2 mRNA transcripts lacking 3′ coding sequences were expressed in all normal and malignant breast tissues examined. These data are preliminary in nature as they report alternate MDM2 mRNA transcripts, and their potential role in normal and neoplastic breast development awaits their cloning and further biochemical analysis. In addition, our results reveal that expression of MDM2 is very complex with multiple RNAs and protein isoforms being expressed in both normal and neoplastic tissues. Reagents that can distinguish the alternate isoforms will be essential to accurately determine the patterns of MDM2 expression.

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