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TITLE: Assessment of mdm2 Alterations on p53 Expression in Breast Cancer

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Assessment of mdm2 Alterations on p53 Expression in Breast Cancer

Mdm2 is part of a complex mechanism that regulates the expression of p53 tumor suppressor. mdm2 mRNA expression was characterized in a cohort of invasive breast cancers and normal breast specimens. Mdm2 reverse-transcription polymerase chain reaction (RT-PCR) products included full-length mdm2 (1526 bp) as well as smaller products (653, 281, 254 and 219 bp). The 653 bp product was an alternatively spliced product, while the 281, 254, and 219-bp mdm2 products were aberrantly spliced. RT-PCR with normal breast RNA samples yielded only the 1526 bp product and a small amount of the 653 bp product. The 653 bp alternatively spliced product was expressed at increased levels in 21% of breast cancers and the smaller aberrantly spliced mRNA products (281 bp, 254 bp and/or 219 bp) were expressed in another 16% of breast cancers. The alternatively spliced mRNAs and aberrantly spliced mRNAs lacked either the entire binding domain for p53 or the majority of the binding domain. An association was demonstrated between expression of aberrantly and/or alternatively spliced mdm2 mRNAs and a lack of progesterone receptor, or the presence of p53 tumor suppressor gene mutations. The presence of aberrant expression products of mdm2 in breast cancer specimens was correlated with a shortened overall patient survival.
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# ANNUAL REPORT

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Introduction.

The p53 gene is important in the pathogenesis of many types of human cancers. p53 is a transacti"vator of WAF1/Cip1 (1) and gadd45 (2) which are involved in arrest of the cell cycle and DNA damage repair, respectively. p53 regulates its own expression through the mdm2 gene (3, 4). Initially, it was thought that overexpression of p53 protein was caused by mutations in the p53 gene; however, since a large review (5) showed that only 22% of tumors had a mutation in the DNA sequence, this contention is the subject of debate (6-8). These data suggest the possibility that a gene which down-regulates p53 such as mdm2, if inactivated, might be responsible for some of the overproduction of p53 protein in the absence of mutations in the gene.

mdm2 is up-regulated by p53 and effects p53 activity in two ways: 1.) it inhibits p53 function by binding to the transactivation site of p53 (4) and 2.) it promotes rapid p53 degradation through a proteosome dependent mechanism. (9, 10). There are two promoters in the mdm2 gene (19). mdm2 transcript can originate from either of those two promoters, P1 and P2, which result in two different 5'-untranslated regions (5'-UTRs). The P1 promoter is upstream of exon 1 and is independent of p53. The P2 promoter is located in intron 1 upstream of exon 2 and is activated in the presence of p53 (19). mdm2 is amplified in approximately 30% of soft tissue sarcomas (11, 12). The overall frequency of mdm2 amplification in human tumors is 7% (3). In human breast cancers, mdm2 amplification is a rare event, and no mutations have been described (14). However, transgenic mice overexpressing mdm2 protein but lacking functional p53 had deranged growth patterns in breast ducts and lobules during pregnancy or lactation. Ten to twenty percent of these mice developed mammary carcinomas after long latency (15). Evidence of different sizes of mRNA transcripts was noted when mdm2 was initially cloned (12) and more recently, altered mRNAs, postulated to be the product of alternative splicing, were described in ovarian carcinomas (16), bladder carcinomas (16) and astrocytic neoplasms (37). Alternatively spliced mdm2 mRNAs were marginally associated in ovarian carcinomas with poor tumor differentiation and late stage (16). Different sized mdm2 proteins have been noted in normal mammary epithelial cells and breast cancer cell lines (17). In this investigation, mdm2 mRNA alterations were analyzed in a cohort of 38 invasive breast cancers and 9 normal breast specimens. Not only was alternatively spliced mdm2 mRNA
identified in these breast carcinomas, but previously undescribed aberrant mdm2 splice products were also identified and characterized. Correlations were made between mdm2 mRNA alterations and established prognostic markers or other molecular markers or clinical outcome in these breast cancers.

Results.

Southern Hybridization. Analysis of genomic DNA from 38 breast cancers and 50 ovarian carcinomas by Southern hybridization showed no amplification of mdm2 in any specimen.

Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR). A nested PCR protocol was used to amplify the full length mdm2 cDNA as described by others with minor modifications as described below. In brief, the full open reading frame of mdm2 (1573 base pairs) was amplified using nested primer pairs. The PCR primers were 5' of the translation start codon and 3' of the translation stop-codon. PCR amplification was performed using 25 μl reactions, containing 1.6 mM MgCl₂, 40 pM of each primer, 1 mM dNTPs, 5 units of Taq DNA polymerase (Promega), and approximately 100 ng of cDNA. Thirty cycles of amplification were performed at 94°C (1 minute), 58°C (1 minute), and 72°C (2 minutes) in a Perkin Elmer 480 thermocycler using a mineral oil overlay. After the external primers were used, a 2 μl aliquot of PCR product was transferred directly to a new PCR reaction tube with the internal primer pair. Reaction temperatures were the same for both primer sets. The products of the second reaction were run through 1.5% agarose gels (SeaKem LE, FMC Bioproducts, Rockland, ME), stained with ethidium bromide and visualized with ultraviolet transillumination.

RT-PCR analysis of mRNA demonstrated the expected full-length 1526 base-pair (bp) mdm2 product in nearly all breast cancer specimens as well as other, smaller mdm2 products in some breast cancer specimens (Figure 1). Thirty-seven of 38 (97%) breast cancers had the full-length RT-PCR product. In addition, eleven of 37 (30%) breast cancers also had at least one smaller PCR product which measured 653 base-pairs, 281 base-pairs, 254 base-pairs, or 219 base-pairs in length. One sample did not have the 1526 base-pair product but did have a 281 and a 219 base-pair product. Six breast cancers (16%) had both a 1526 base-pair and 653 base-pair products, four breast cancers (10.5%) had 1526 base-pair and 219 base-pair products and one breast cancer (2.5%) had a 1526 base-pair, a 281 base-pair and a 219 base-pair product.
Figure 1. *mdm2* alterations in invasive breast cancers and normal breast tissue.

An ethidium bromide stained 1% agarose gel shows RT-PCR products derived from invasive breast cancers (1-18) and normal breast epithelium (N). Samples 1-18 are invasive breast cancer; samples labeled N1-N4 are normal breast tissue. “L” indicates the lanes loaded with the 1 kb DNA ladder (Gibco/BRL).

Because of the PCR products less than 1526 base-pairs in length, we decided to evaluate normal breast tissue specimens to determine if any of the smaller products were the result of alternative splicing of the predicted mRNA. Nine normal breast tissue specimens were analyzed by RT-PCR for *mdm2* expression products and all were found to contain the 1526 base-pair product. Four of the nine normal breast specimens also contained a 653 base-pair PCR product (Figure 1). However, the 653 base-pair product was expressed at a much lower level than the 1526 base-pair product.

DNA sequence analysis was performed to determine the identity of the various PCR products (Figure 2). The 1526 base-pair product was confirmed as full-length *mdm2*. The 653 base-pair product proved to be an alternatively spliced *mdm2* in which exon 3 (the exon containing the ATG-start site) was spliced in-frame to exon 12 (the exon containing the termination site). DNA sequence analysis of the other, smaller PCR products confirmed that each contained *mdm2* sequences but also demonstrated that each was an aberrant splice variant of *mdm2*. The aberrant splice sites occurred within exons (Figure 2), not at exon-intron boundaries as is usually observed with alternatively spliced products.
A schematic summary is provided comparing the full-length \textit{mdm2} cDNA with each of the RT-PCR products which were isolated and sequenced. The functional domains are identified at the top of the figure.

The 281 base-pair aberrantly spliced \textit{mdm2} product consisted of 204 bases of 5' \textit{mdm2} open reading frame from exons 3 and 4 spliced to 50 bases from exon 5 and 76 bases from exon 12. The 219 base-pair aberrantly spliced \textit{mdm2} product consisted of 155 bases from exons 3 and 4 spliced to the first 16 bases of exon 5 followed by the last 48 bases of exon 12. The pattern of splicing in these \textit{mdm2} fragments was of interest because the splice donor and splice acceptor sites were in regions of exact sequence homology within the exons that were spliced.

The 254 base-pair aberrantly spliced \textit{mdm2} product consisted of 141 bases from exons 3 and 4 spliced to a 14 base-pair insertion with no known homology, followed by 99 bases from exon 12. The introduction of this 14 base-pair insertion, possibly from unspliced intron sequences, results in a predicted change in the open reading frame.

In each of these small, aberrantly spliced \textit{mdm2} products the p53 binding site, the nuclear localization signal, the acidic domain and a portion of the RING finger were all deleted from the mRNA expression product. The loss of these domains is predicted to effect the function of the \textit{mdm2} product.
Because *mdm2* has both a p53-dependent and a p53-independent promoter, RT-PCR was performed to evaluate promoter usage in these breast cancer cases. The location of the PCR primers O1, O2 and O3, relative to exons 1, 2 and 3 are illustrated as well as the predicted sizes of the PCR products expected from alternative splicing of the mRNA products derived from both promoters (Figure 1B). A 349 bp product is expected with P1 promoter activity due to direct splicing of exon 1 to exon 3. This pattern was observed in mRNA from 31 breast cancers. Both a 349 bp product (exon 1-exon 3) and a 150 bp product (exon 2-exon 3) expected from P2 promoter activity in mRNA from 17 breast cancers. Only a 270 bp PCR product, confirmed by direct DNA sequence analysis as exon 2, intron 2 and exon 3, was observed in mRNA from 5 breast cancers, consistent with P1 promoter activity without removal of intervening intronic sequence. Neither P1 nor P2 promoter usage was selectively associated with alternatively or aberrantly spliced *mdm2* transcripts.

**Figure 3. Schematic Diagram of 5’ untranslated transcripts from *mdm2***

![Schematic Diagram of 5’ untranslated transcripts from *mdm2*](image)

Schematic diagram showing the position of PCR primers O1, O2 and O3 relative to the 5' genomic *mdm2*. Three types of mRNA transcripts (350 bp, 150 bp and 270 bp) were identified with these primers, one (349 bp) derived from promoter 1 (P1) activity and two (150 bp and 270 bp) derived from promoter 2 (P2) activity. The 350bp PCR product from P1 activity is known to
be p53 independent and lacked exon 2 sequences (presumably due to mRNA splicing) but contained exon 1 sequence (as part of the 5’ UTR) and exon 3 sequence. The 150 bp PCR product lacked exon 1 sequence but had exon 2 and exon 3 sequences. The 270 bp PCR product also lacked exon 1 sequence but contained exon 2, intron 2 and exon 3 sequence. This 270 bp product was described by Sigalas (16).

The presence or absence of alternate and aberrant splicing products of the \textit{mdm2} gene was compared with expression of p53, HER2/neu (c-erbB2), estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor (EGFR), and with DNA ploidy status (Table 1). The presence of p53 overexpression, HER2/neu overexpression, ER, PR and EGFR were assessed with immunohistochemistry. DNA ploidy was assessed with computerized image analysis. These results are summarized in Table 1.
Table 1: Summary of the Associations between Clinical Markers and *mdm2* Expression

<table>
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NA= tissue not available for analysis
Mdm2 alternative and aberrant splicing had statistically significant correlations with clinical parameters. mdm2 expression was considered to be normal when either a single 1526 base pair RT-PCR product was identified or when a 1526 base product and a 653 base product were identified. The presence of abnormal mdm2 splice variants (281, 254, or 219 bp products) was correlated with lack of PR (p=0.030) and with p53 mutations (p=0.02). The presence of aberrant mRNAs were not associated with ER expression (p=0.395), p53 overexpression (p=0.624), HER2/neu overexpression (p=0.827), EGFR expression (p=0.3) or DNA aneuploidy (p=0.643) (Table 1).

If the 1526 base pair full length product was considered normal, and the 653, 281, 254, and 219 base fragments were considered abnormal, the expression of abnormal mdm2 fragments correlated differently with clinical markers. There was still no correlation with EGFR expression, HER2/neu overexpression or DNA ploidy status; however, lack of ER expression showed a trend but did not reach statistical significance (p=0.087). Abnormal mdm2 fragments did correlate with low PR expression (p=0.036) and p53 immunopositivity (p=0.023). Variant mdm2 alterations of any kind were significantly correlated with p53 mutations (p=0.0003), in contrast to the less significant association (p=0.02) observed when the 653 bp product was considered a normal variant. This change was accounted for, in the main, by the fact that four of the eight samples containing a 653 bp RT-PCR product had a mutation in the p53 gene.

Comparison of the presence or absence of altered mdm2 mRNAs to clinical outcome showed a correlation between the presence of abnormal RT-PCR products and outcome. Assessing the 653 bp RT-PCR products and RT-PCR products less than 300 bases as abnormal, no correlation between outcome and the presence or absence of the altered mdm2 mRNAs was noted. However, when only RT-PCR products less than 300 bp (281, 254, 219 bp) were considered abnormal, a highly statistically significant correlation was seen between expression of these RT-PCR products and decreased overall survival (p=0.0036) (Figure 4)

Key Research Accomplishments.
1. Identification of alternative splicing and aberrant splicing of mdm2 in breast cancer.
2. Association of aberrantly spliced transcripts in breast cancer tissue was associated with shortened overall survival.
Reportable Outcomes.


**Conclusions.**

*mdm2* mRNA is altered in approximately 30% of human breast cancers. The altered expression involved loss of functional domains interacting with p53 suggesting that *mdm2* may play an important role in derangement of the p53 pathway in some breast cancers. The presence of aberrantly spliced products was correlated with the presence of poor prognostic markers and with shortened overall patient survival, indicating that the aberrant splicing of *mdm2* may have clinical significance.

**References.**


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D. Bibliography


Alternative and Aberrant Messenger RNA Splicing of the mdm2 Oncogene in Invasive Breast Cancer

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ABSTRACT

mdm2 is part of a complex mechanism that regulates the expression of p53 as well as the function of Rb, p19ARF, and other genes. In humans, mdm2 dysregulation is associated with gene amplification. This study was undertaken to characterize altered mdm2 expression in a cohort of 38 invasive breast cancers and 9 normal breast specimens. Reverse-transcription PCR with primers spanning the entire open reading frame of the mdm2 gene in breast tissue RNA samples generated PCR products of full-length mdm2 (1526 bp) as well as smaller products (653, 281, 254, and 219 bp). Sequence analysis demonstrated that the 653-bp product was an alternatively spliced product (defined as splicing at the exon/intron boundary consensus sites), whereas the 281, 254, and 219 mdm2 products were aberrantly spliced products (splicing at sites not considered to be exon/intron boundary sites). Reverse-transcription-PCR with normal breast tissue RNA samples yielded only the 1526-bp product in five samples and the 1526-bp product and the 653-bp product in four samples. The 653-bp alternatively spliced product was expressed in 21% of breast cancers, and the smaller, aberrantly spliced mRNA products (281 bp, 254 bp, and/or 219 bp) were expressed in 16% of breast cancers. The protein products predicted by the alternatively spliced mRNAs and the aberrantly spliced mRNAs lacked either the entire binding domain for p53 or the majority of the binding domain for p53. Immunohistochemical analysis of HER2/neu (c-erbB2), estrogen receptor, progesterone receptor, epidermal growth factor receptor, and p53 protein was performed. p53 sequence alterations were identified by mismatch detection and confirmed by p53 oligonucleotide microarray technology. An association was demonstrated between the expression of aberrantly and/or alternatively expressed mdm2 mRNAs and a lack of progesterone receptor. An association was also demonstrated between mdm2 aberrantly and/or alternatively expressed products and the presence of p53 tumor suppressor gene mutations. mdm2 is transcribed from two different promoters: one, p53-dependent, and the other, p53-independent. The 5′ untranslated region of the transcripts was evaluated to determine the promoter usage in each breast cancer specimen. No correlation was observed between mdm2 splice products and promoter usage. The presence of aberrant expression products of mdm2 in breast cancer specimens was correlated with a shortened overall patient survival. These observations suggest that mdm2 expression is altered in invasive breast cancer and is associated with more aggressive disease.

INTRODUCTION

The p53 gene is important in the pathogenesis of many types of human cancers. p53 is a transactivator of WAF1/Cip1 (1) and gadd45 (2), which are involved in arrest of the cell cycle and DNA repair, respectively. p53 regulates its own expression through the mdm2 gene (3, 4). Initially, it was thought that overexpression of p53 protein was caused by mutations in the p53 gene; however, since an extensive review (5) showed that only 22% of tumors had a mutation in the DNA sequence, this contention has been the subject of debate (6–8). These data suggest the possibility that a gene that downregulates p53, such as mdm2, if inactivated, might be responsible for some of the overproduction of p53 protein in the absence of mutations in the gene.

mdm2 is up-regulated by p53 and effects p53 activity in two ways: (a) it inhibits p53 function by binding to the transactivation site of p53 (4); and (b) it promotes rapid p53 degradation through a proteosome-dependent mechanism (9, 10). mdm2 is amplified in ~30% of soft tissue sarcomas (11, 12). The overall frequency of mdm2 amplification in human tumors is 7% (13). In human breast cancers, mdm2 amplification is a rare event, and no mutations have been described (14). However, transgenic mice overexpressing mdm2 protein but lacking functional p53 had deranged growth patterns in breast ducts and lobules during pregnancy or lactation. Ten to 20% of these mice developed mammary carcinomas after long latency (15). Evidence of different sizes of mRNA transcripts was noted when mdm2 was initially cloned (12), and more recently, altered mRNAs, postulated to be the product of alternative splicing, were described in ovarian carcinomas and bladder carcinomas (16) and in astrocytic neoplasms (17). Alternatively spliced mdm2 mRNAs were marginally associated (P = 0.085) with poor tumor differentiation and late stage in ovarian carcinomas (16). Different-sized mdm2 proteins have been noted in normal mammary epithelial cells and breast cancer cell lines (18). In this study, mdm2 mRNA alterations were analyzed in a cohort of 38 invasive breast cancers and 9 normal breast specimens. Not only was alternatively spliced mdm2 mRNA identified in these breast carcinomas, but previously undescribed aberrant mdm2 splice products were also identified and characterized. Correlations were made between mdm2 mRNA alterations and established prognostic markers or other molecular markers or clinical outcome in these breast cancers.

MATERIALS AND METHODS

Tissues. The use of human tissue in this investigation was reviewed and approved by the University of Southern California Institutional Research Committee. Thirty-eight frozen invasive ductal breast carcinomas and nine normal breast tissue specimens, obtained from storage at −186°C in a liquid nitrogen freezer of the University of Southern California Breast Tumor and Tissue Bank, were used for these investigations. Frozen tissue sections stained with H&E were used to confirm the histological composition of the specimens.

Total RNA Isolation. Total RNA was extracted from 10–20 serially cut, 10-µm thick, frozen tissue sections using TRIzol® (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. The RNA was precipitated using isopropanol, washed once with 70% ethanol, and allowed to dry at room temperature. The RNA was dissolved in 30 µl of RNase-free H2O, and the absorbance was measured. The RNA was then analyzed for the integrity of the 28S, 18S, and SS rRNA bands by Northern hybridization using formaldehyde containing agarose gels.

Reverse Transcription. Mouse mammary leukemia virus reverse transcriptase (MMLV-RT; Life Technologies, Inc.) was used for reverse transcrip-
tion to produce cDNAs according to the following protocol. Two μg of total RNA was used in each reaction to ensure a representation of all mRNAs. Total RNA was mixed with 0.5 μg of oligo dT 15 primer, brought up to a total of 7 μl using RNase-free H2O, heated to 70°C for 10 min, and immediately afterward iced for 5 min. The RNA and oligo dT 15 (Promega, Madison, WI) were added to 13.5 μl of reverse transcriptase solution (40 units of RNAguard; Pharmacia, Piscataway, NJ), 1X First Strand buffer (Life Technologies, Inc.), 1.0 mM dNTPs triphosphates (Promega), 0.125 mM MgCl2 (Promega), and incubated at 37°C for at least 1 h. After cDNA synthesis, the samples were stored frozen at −80°C.

_mdm2_ Primer Design and PCR. A nested PCR protocol was used to amplify the full-length _mdm2_ cDNA as described by Sigalas et al. (16), with the modifications detailed below. In brief, the entire open reading frame of _mdm2_ (1266 bases) was amplified using the following nested primer sets: 

- A) external primer pair: sense, 5'-CTGGGGAGTCTTGAGGGACC-3', and antisense, 5'-CAGGTTGCTTAATCTTCTAG-3'; and 
- B) internal primer pair: sense, 5'-CCGCAAAACCCCGGGCACGCAAATGTGCA-3', and antisense, 5'-CTCTTATAGACAGGTCAACTAG-3' (Fig. 1A).

PCR amplification was performed with 25-μl reactions using 1.6 mM MgCl2, 0.125 μM each primer, 1 mM dNTPs, and 1X Taq polymerase (Promega), and 100 ng of cDNA. Thirty cycles of 94°C (1 min), 58°C (1 min), and 72°C (2 min) were performed in a Perkin-Elmer 480 using a mineral oil overlay. After the external primers were used, a 2-μl aliquot was transferred directly to a new reaction tube for PCR using the internal primers. Reaction temperatures were the same for both primer sets. The products of both reactions were run through 1.5% agarose gels (SeaKem LE; FMC Bioproducts, Rockland, ME) with ethidium bromide and visualized by UV transillumination.

The only difference between the 653-bp _mdm2_ product described later and the 707-bp product characterized by Sigalas et al. (16) is the reference numbering. Our 653-bp product refers to the number of bps counted between the start and stop codons, but does not include the PCR primers. The 707-bp numbering as described by Sigalas et al. (16) includes the PCR primers, which are outside of the start and stop codons.

_mdm2_ contains two promoters, one (P1) upstream of exon 1, which is _p53_-independent, and another (P2) upstream of exon 2, which is _p53_-dependent. To distinguish transcripts expressed by each of these two promoters, two different _5'_ primers, located in exon 1 (primer O1-5'-CTGTTGGTGCG-GAAAGATGG 3') and exon 2 (primer O2-5'-TGTGGTAGCTGGCGATT-GGA 3'), respectively, were used with a single _3'_ primer located in exon 3 (primer O3-3' TCTCTTGTGCGGAGCTGG 3') as described by Zauberman et al. (19; Fig. 1B).

PCR was also used to assess the _mdm2_ gene for potential deletions in the genomic DNA. Primers were designed to span 1566 bases from the start of exon 5 into the 3'-UTR. These primers were: (a) sense, 5'-TTCTTTTTTTACTCTTGAGCCAG-3'; and (b) antisense, 5'-TCTCATATATTAGTAG-3' (Fig. 1A).

_mdm2_ Product Cloning and DNA Sequencing. Each breast cancer containing PCR products other than the full-length (1526 bp) and 653-bp sizes were either cloned using the TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced directly or sequenced or sequenced and directly sequenced from the PCR products. For cloning and sequencing, cDNAs were PCR-amplified from the _mdm2_ internal primers to generate sufficient product. The product was then purified with Qiagen PCR purification columns (Qiagen, Chatsworth, CA) and ligated into the pCR.1 vector. After transformation, the plasmid was isolated (Wizard plasmid mini-preps; Promega) and sequenced using the ThermoSequenase kit (Amersham, Arlington Heights, IL), according to the manufacturer's instructions. Approximately 250 ng of plasmid were used for sequencing. Alternatively, products were gel-purified using the Qiaquick Gel Extraction Kit (Qiagen) and sequenced directly using the ThermoSequenase Kit.

Analysis of Mutations in _p53_. In all 38 cases, the _p53_ gene was analyzed by the Mismatch Detect kit in conjunction with the _p53_ cDNA screening module (Ambion, Austin, TX) according to the manufacturer's instructions. Briefly, cDNAs were amplified with a nested PCR protocol using external and internal primers specific to _p53_. These primers span the evolutionarily conserved regions of _p53_ from codon 91 to codon 368. PCR amplification was carried out using a three-temperature protocol as follows: (a) 30 s at 94°C to denature; (b) 30 s at 55°C to anneal the primers; and (c) 40 s at 72°C for extension. Samples were amplified for 30 cycles before 5 min at 72°C. The internal primers were designed with a T7 RNA polymerase promoter on the 5' end of the sense promoter and a SP6 RNA polymerase promoter on the 5' end of the antisense promoter. The appropriate RNA polymerase was added to the internal primer PCR amplification product, and subsequently the RNA from either promoter was hybridized to a complementary wild-type _p53_ RNA. After hybridization, the samples were digested using various RNases, which cleaved the RNA at the sites of mismatched bps. The digested samples were analyzed on 1.5% agarose gels (FMC).

Breast cancers identified as having _p53_ sequence alterations by mismatch detection, as described above, were characterized additionally for specific alterations in the bcl2, bcl-xl, bcl-2, and mdm2 genes.
sequence alterations using p53 oligonucleotide microarrays (p53 GeneChip; Affymetrix, Santa Clara, CA) as described elsewhere in detail (20). In brief, genomic DNA from exons 2–11 was amplified with PCR primers based on flanking intronic sequences to permit analysis of each intron/exon splice junction. Each sample DNA was then fragmented with DNase, labeled with Fluorescein-N6-deoxATP (DuPont NEN; Boston, MA) by way of a terminal transferase reaction and hybridized to a p53 GeneChip Array. Fluorescently labeled fragmented DNA samples were washed over the chip and allowed to bind to complementary oligonucleotide probes. Hybridized probe arrays were then read using the GeneArray Scanner (HP G2500A; Hewlett-Packard). As a quality assurance step, a control oligonucleotide was added to each sample during hybridization to examine the signal intensity and proper alignment of the probe array after the scan. Before the collection of image data, the scanner confirmed the correct position and alignment of the chip by focusing on a series of defined positions.

To account for any variations that occurred during the assay, each sample batch was processed with human placental DNA as a wild-type control (Sigma; St. Louis, MO). Any sequence mismatch present in sample DNA was identified by comparison to the control placental DNA.

Immunohistochemistry. The peroxidase/antiperoxidase technique was used to identify various protein products in tissue sections. Frozen sections 4 µm thick were cut and fixed appropriately and treated with primary antibody for 1 h at room temperature. Secondary and tertiary antibodies were applied for 0.5 h at room temperature. Each slide was stained with ethyl green, a nuclear counterstain.

p53 protein was localized with the antihuman p53 mouse monoclonal antibody DO-7 (Dako Corporation, Carpinteria, CA) at a 1:100 dilution in 10% normal goat serum. Because membrane staining is common in normal epithelial cells, only membrane staining in the carcinoma was considered positive.

Altered mdm2 mRNAs in Normal Breast Tissue. PCR analysis of the reverse transcribed mRNA revealed the presence of the expected full-length product (1526 bp) as well as other, smaller, products (Fig. 2). Thirty-eight cases (34%) also had smaller RT-PCR products, which were 653, 281, 254, and/or 219 bases in length. One sample did not have the full-length 1526-bp RT-PCR product but had products of only 281 and 219 bp in length. Six (16%) samples had 1526- and 653-bp products, three samples (8%) had 1526- and 219-bp products, one sample (2.5%) had 1526-, 281-, and 219-base products, and one sample (2.5%) had 1526- and 254-bp fragments.

Sequence analysis was carried out to determine the composition of the altered products. The 653-bp fragment was an alternatively spliced mdm2 mRNA. The exon 3 sequence was directly associated with the exon 12 sequence at the intron/exon boundary splice sites without a sequence from any intervening exon (26). The truncated mRNA was in-frame. This fragment was missing a large central portion of the mRNA (Fig. 3, mdm2–653 bp), including 90% (81 of 90) of the amino acids of the 3′ end from the p53 binding domain and the entirety of the nuclear localization signal and acidic domain. The zinc finger and RING finger domains remained intact. This product was identical to a similar product described previously and referred to as mdm2-B (16). Again, the only difference between the 653-bp mdm2 product described and the 707-bp product is the reference numbering. Our 653-bp product refers to the number of bps counted between the start and stop codons but does not include the PCR primers. The 707-bp numbering as described by Sigalas et al. (16) includes the PCR primers, which are outside of the start and stop codons.

The two 281-bp and the five 219-bp fragments appeared to be aberrantly spliced products with the splice donor and acceptor sites in regions of exact sequence homology, which was identified in more than one exon (Fig. 4). The 281-bp products consisted of 205 bases of 5′ mdm2 open reading frame sequence, including exons 3 and 4, and 50 bases of exon 5 spliced into the 3′ 76 bases of exon 12. On the basis of this sequence analysis, the predicted amino acid sequence would be in-frame beyond the cryptic splice site. The 281-bp product

RESULTS

mdm2 mRNAs in Normal Breast Tissue. The normal ductal or lobular epithelium from nine normal breast samples (derived from reduction mammoplasties) were analyzed to clarify normal expression of mdm2. PCR analysis of the reverse-transcribed mRNAs from nine samples of normal breast tissue revealed the presence of the expected full-length RT-PCR product (1526 bp) in every case as well as 653-bp RT-PCR product in four of nine cases (Fig. 2, N1 and N4). The 653-bp product had a weaker intensity in normal epithelium compared with breast carcinomas (Fig. 2; compare Lane 13 with N1 and N4).

Altered mdm2 mRNAs in Tumor Tissues. PCR analysis of the reverse transcribed mRNA revealed the presence of the expected full-length product (1526 bp) as well as other, smaller, products (Fig. 2). Thirty-seven breast cancers (97%) had a full-length, 1526-bp RT-PCR product. Thirteen of 38 samples (34%) also had smaller RT-PCR products, which were 653, 281, 254, and/or 219 bases in length. One sample did not have the full-length 1526-bp RT-PCR product but had products of only 281 and 219 bp in length. Six (16%) samples had 1526- and 653-bp products, three samples (8%) had 1526- and 219-bp products, one sample (2.5%) had 1526-, 281-, and 219-base products, and one sample (2.5%) had 1526- and 254-bp fragments.
was missing 43% of the p53 binding domain, the entire nuclear localization signal, acidic domain, and zinc finger, as well as over half of the RING finger domain (Fig. 3, mdm2-281 bp). In the 281-base fragment, the new, cryptic donor site of exon 5 ended with a 6-base sequence of AGAAAGC, and the same sequence was observed at the acceptor site in exon 12 (Fig. 4A). Thus the splice donor sequence within exon 5 was 517-AGAAAGC/AAGAC-524 and the splice acceptor sequence within exon 12 was 1708-AGAAAGC/TAAAGA-1719 (Underlined sequence indicates exact sequence match, uppercase letters denote coding bases). This same 6-bp sequence occurs at four different sites in the coding sequence of mdm2 (Fig. 4A). A potential cryptic splice site was identified within this repeated 6-bp sequence (AG-AAGC). The aberrant, truncated mRNA is predicted to have the same open reading frame as full-length mdm2 mRNA.

The 219-bp fragment also appeared to be the product of similar aberrant splicing involving cryptic splice sites. This product consisted of exon 3, exon 4, and the first 16 bases of exon 5 spliced into the last 48 bases of exon 12 (Fig. 3, mdm2-219 bp). The splice site varied among the 10-base site, TGCCAGTAT, in exon 5 and a 10-base site, TGCCCATAT, in exon 12. This same sequence occurs only at these two sites in mdm2, where, apparently, aberrant splicing occurred at cryptic splice sites (CCAG-TAT; Fig. 4B). Four of five cases were spliced within the overlap region, and consequently, the exact location of the splice could not be ascertained. One of the cases was spliced 2 bp outside of the overlap region. On the basis of this sequence analysis, we expect the final protein product of the 219-bp mRNA to be out-of-frame beyond the aberrant splice site.

The 254-bp product had a different splicing arrangement. In a single case, the amplified mRNA contained the entirety of exon 3 and exon 4 followed by the last 99 bases of exon 12. At the splice junction between the exon 4 and exon 12 sequences was a 14-bp insertion, GTGAAACTCCATCT, which had sequence homology to intrinsic sequences. The predicted final protein product would be expected to be frame-shifted out of the original reading frame (Fig. 3, mdm2-254 bp).

In each of these smaller fragments, the majority of the mdm2 mRNA was removed. In general, more than one-half of the p53 binding site was removed, as well as the entire nuclear localization signal, acidic domain, and a portion of the RING finger.

PCR Analysis of mdm2 Genomic Sequence. The truncated mdm2 mRNAs might possibly be caused by genomic deletions in the mdm2 gene. In studies of yeast (27), a mutated DNA polymerase (pol3-t) created a mutator phenotype that deleted segments of DNA between 3- to 7-bp-long direct repeats, mimicking the results found in some of the mdm2 mRNAs. To investigate the possibility that genomic deletions in mdm2 might give rise to truncated mRNAs, PCR was used to amplify the genomic DNA across the direct repeats where the deletion was predicted. No deletions were noted in the genomic DNA from any of the 38 samples of invasive breast cancer (data not shown).

Mutations around the intron and exon boundary will increase the possibility of splicing at cryptic sites. The genomic sequence flanking
The intron/exon boundary area were sequenced in the aberrant splicing cases, but no mutations were detected.

Identification of mdm2 mRNA 5'-UTRs Corresponding to Transcripts from the p53-independent PI promoter and the p53-dependent P2 promoter. The nested PCR primers used above to span the transcript from exon 3 to exon 12 permitted an assessment of the size and sequence of the mdm2 open reading frame. However, mdm2 transcript can originate from either of two different promoters, PI and P2, which result in two different 5'-UTRs. The PI promoter is upstream of exon 1 and is independent of P2 (19). The transcript originating from the PI promoter has exon 1 spliced directly to exon 3, eliminating exon 2 sequences from the final product. The transcript originating from the P2 promoter lacks exon 1 sequences beginning with exon 2, which is spliced to exon 3. The presence or absence of each 5'-UTR was determined with specific PCR primers (Fig. 1B) to assess mdm2 promoter use for each breast cancer specimen.

The location of O1, O2 and O3 PCR primers relative to exons 1, 2, and 3 are illustrated, as well as the predicted sizes of the PCR products expected from the alternative splicing of the mRNA (Fig. 1B). A 350-bp product expected from the splicing of exon 1 to exon 3 without detection of the 1526-bp product predicted from the splicing of exon 2 to exon 3 was observed in mRNA from 31 breast cancers. Both a 350-bp product (exon 1-exon 3) and a 150-bp product (exon 2-exon 3) were observed with these primers in mRNA from 17 breast cancers.

Only a 270-bp PCR product, confirmed by direct DNA sequence analysis as exon 2, intron 2, and exon 3, was observed in mRNA from 5 breast cancers. Analysis of three breast cancers was not performed because of insufficient mRNA. There were no statistically significant associations between mdm2 alternative and aberrant splicing products and promoter usage.

Correlation of Altered mdm2 mRNAs with p53 Mutations. The presence of p53 mutations was assessed using Mismatch Detection. These alterations were additionally characterized by p53 oligonucleotide microarray analysis of genomic DNA from these cases. Seventeen cases were identified as having p53 sequence alterations by mismatch detection. Of these 17 alterations, 9 cases were confirmed as mutated by DNA chip analysis, 3 were reconsidered to be wild type, 3 were not checked because of a lack of DNA, and 2 were found to be polymorphisms. The results are summarized in Table 1.

Correlations of Altered mdm2 mRNAs with Prognostic Markers and Clinical Outcome. The presence or absence of alternate and aberrant splicing products of the mdm2 gene was compared with the expression of p53, HER2/neu (c-erbB2), ER, PR, and EGFR, and with DNA ploidy status (Table 1). The presence of p53 overexpression, HER2/neu overexpression, ER, PR, and EGFR were assessed with immunohistochemistry. DNA ploidy was assessed with computerized image analysis. These results are summarized in Table 2.
Comparison of the presence or absence of altered \textit{mdm2} mRNAs with clinical outcome showed a correlation between the presence of abnormal RT-PCR products and outcome. Assessing the 653-bp RT-PCR products and RT-PCR products <300 bases as abnormal, no correlation between outcome and the presence or absence of the altered \textit{mdm2} mRNAs was noted. However, when only RT-PCR products <300 bp (281, 254, and 219 bp) were considered abnormal, a highly statistically significant correlation was seen between expression of these RT-PCR products and decreased overall survival (\( \geq 0.0036 \); Fig. 5).

**DISCUSSION**

Relatively little is known about the alternative and aberrant splicing of \textit{mdm2} mRNA. Alternative splicing of \textit{mdm2} transcripts has been described in glioblastomas (28) and astrocytomas (17) as well as in ovarian and bladder carcinomas (16). Five different alternatively spliced \textit{mdm2} transcripts, referred to as \textit{mdm2-a}, \textit{mdm2-b}, \textit{mdm2-c}, \textit{mdm2-d}, and \textit{mdm2-e}, have been described previously by Sigalas et al. (16). We report here the discovery of three previously undescribed transcripts, which are 281 bp, 254 bp, and 219 bp in size, as well as a 653-bp transcript that is identical to \textit{mdm2-b}. None of the transcripts reported previously were the result of splicing at cryptic splice sites within \textit{mdm2} exons as described by us here. In addition, the relationship of these truncated transcripts to either the P1 p53-independent or the P2 p53-dependent promoters was not investigated previously.

Three studies in breast cancer report that multiple \textit{mdm2} proteins are produced, but they disagree about the size of the proteins that are normally and abnormally expressed (18, 29, 30). One study showed that multiple mRNAs exist in both tumor and normal breast tissues (30), whereas another study showed that low-molecular-weight proteins predominate in benign epithelium (18). In contrast, the last study of matched benign breast epithelium and breast tumor found that a 57,000 protein (the "normal" size of the \textit{mdm2} protein is \( M, 90,000 \)) was more highly expressed in neoplastic tissues, which suggested that \textit{mdm2} expression is altered during progression (29). The present work shows that \textit{mdm2} expression in some breast cancers is characterized by truncated mRNAs <300 bases long. Less than full-length transcripts of \textit{mdm2} have been described as being associated with ovarian carcinomas (16), astrocytomas (17), glioblastomas (28), and leukemias (31).

However, our results also revealed expression of the alternatively spliced 653-bp RT-PCR product in normal tissues, although at an apparently lower level than found in the carcinomas. Results presented here show that \textit{mdm2} is alternatively spliced in normal breast epithelium and is both alternatively and aberrantly spliced in invasive breast cancers. Normal and neoplastic tissues showed differences in both the pattern of altered splicing and in the amounts of altered products.

Which splice products are considered normal and which are abnormal? "Alternative" splicing makes use of inherent intron-exon splice sites of a single mRNA transcript to produce different mRNAs through differential splicing. Alternative splicing is a common event in a number of different genes and appears to be a normal process by which a single gene can produce different proteins with different functions. For instance, the \textit{adenosin deaminase polyposis coli} gene, a tumor suppressor gene known to be altered early in colon carcinogenesis, is normally expressed both with and without exon 1 in the brain, heart, and skeletal muscle in humans and mice, producing novel amino terminal proteins. Thus, \textit{adenosin deaminase polyposis coli} is alternatively spliced and creates different proteins in response to tissue-dependent signals as well as signals to differentiate (32). Alternative splicing also appears to be important in generating the cell-specific heterogeneous membrane proteoglycans that make up the family of proteins generated from the CD44 gene. The heterogeneity is derived from alternative splicing as well as posttranslational modification in 1-10 variant exons that encode parts of the extracellular domain (33). Certain cancers demonstrate dysregulation of alternative splicing (33). Human thyroid and breast cancers showed novel CD44 mRNAs with large coding sequence deletions flanked by short, direct sequence repeats (33, 34).

In contrast with alternative splicing, "aberrant" splicing is the splicing of mRNA that is misdirected and does not occur at \textit{de facto} splice sites. \textit{TSG101} gene and \textit{FHIT} gene mRNAs show evidence of both aberrant and alternative splicing. \textit{TSG101}, a putative tumor suppressor gene that was originally thought to be mutated in breast cancers (35, 36), was found on reexamination to be subject to aberrant splicing that mimicked the original "mutations" (37, 38). These aberrations were predominantly truncating alterations at a discernibly normal splice junction. The \textit{FHIT} gene, another putative tumor suppressor gene, similarly shows evidence of both aberrant and alternative splicing in breast (39), head and neck (40), and lung tumors (41). Specifically in lung cancer, the splicing of \textit{FHIT} is characterized both by losses of individual exons. loss of exons with insertions of un-
known DNA sequences, and losses of whole portions of mRNA, without regard for proper splice junctions (41). In both genes, evidence indicates that alternative splicing is a relatively common event not necessarily associated with malignancy; in contrast, aberrant splicing appears to be associated with malignancy.

The pattern of deletions between distant short direct repeats, such as those characterized in the 281- and 219-bp transcripts, was similar to data obtained from a study of a mutant DNA polymerase in Saccharomyces cerevisiae (27). In that study, a mutation of DNA polymerase δ (called pol 3–t) caused an ~1000-fold increase in 7- to 61-bp deletions between direct repeat sequences, possibly through a mechanism of replication slippage (42). To assess our cases for the effects of a similar DNA polymerase δ mutation, PCR was used to amplify genomic DNA across the potentially deleted regions. No deletion of genomic sequence between repeat sequences was detected. Whereas relaxation of mRNA splicing fidelity has been suggested as a mechanism for the aberrant splicing noted in both the TSG101 (37) and FHIT (38) genes, the specific mechanism of sequence excision between short direct repeats has been described previously only in the CD44 gene in breast and thyroid cancers. It seems possible that an RNA polymerase may play an important role in the generation of these deletions in mdm2 mRNAs, possibly through an undescribed mechanism of "transcription" slippage.

Alternatively, on the basis of our findings of aberrant splicing at direct repeat sequences containing potential cryptic splice donor and acceptor sites, it seems more likely that these cryptic splice sites are selected because of either a mutation at the original splice site or because of changes in the splicing machinery or splicing efficiency. Sequence analysis of the aberrantly spliced products demonstrated that splicing occurred between direct repeat sequences (Fig. 4). Each of these repeat sequences contained sites that might serve as cryptic splice sites (Fig. 4). In the 281-bp mdm2 transcript, AG-A in the first direct repeat may serve as the donor site for splicing to AG-A in the last direct repeat sequence, which might have served as the acceptor site. In the 219-bp product, AG-T in the first direct repeat sequence may have served as the donor site for an AG-T acceptor site in the second oligonucleotide repeat. These potential cryptic donor and acceptor sites have sequences at exon-intron-exon donor-splice-acceptor sites that have been described previously (43).

The presence or absence of aberrant splicing was correlated with clinically useful information. Considering both alternative and aberrant splice products of mdm2 to be abnormal, there were statistically significant correlations with a lack of PR expression, p53 overexpression, and with the presence of p53 mutations. However, there was no correlation with overall survival. Also notable is the trend (P = 0.087) toward a lack of ER expression in the presence of alternative and aberrant mdm2 splicing. Studies have suggested that ER expression is associated with mdm2 overexpression as detected by Northern and Western blots as well as by immunohistochemistry (18, 44), but this correlation was not observed in another study (29). However, a comparison is not applicable in the current study because the differences in the types of mRNAs and in posttranslational mRNA processing have been assessed and not the quantity of mdm2 transcripts expressed.

The presence or absence of mdm2 alternative and/or aberrant splicing was correlated with clinically useful information. Previous studies showed that low expression of ER and PR (45) as well as overexpression of p53 protein (46) and the presence of p53 mutations (46–48) correlate with decreased survival in breast cancer. In this study, we found that aberrant (281-, 254-, and 219-bp) and alternative mdm2 (653-bp) splice products were significantly correlated with low PR expression (P = 0.036), p53 overexpression (P = 0.023), and p53 mutations (P = 0.0003). There was no correlation with survival in this analysis. However, when only aberrant splicing was analyzed, and both 1526-bp and 653-bp are considered normal, we found that aberrant splicing was strongly associated with shorter overall survival (P = 0.0002; Fig. 5). Thus, these results reveal both correlations with clinical markers and a strong association with poor clinical outcome. To our knowledge, this is the first study to correlate the presence of alternative or aberrant mdm2 transcripts with a shorter overall patient survival in breast cancer. A more critical assessment of mdm2 as a potential marker of poor clinical outcome will require the analysis of a larger series of cases.

The connection between aberrant splice products (281, 254, and 219 bp) and poor clinical outcome may involve RNA-binding. Recent evidence indicates that the RING finger domain is necessary for the binding of mdm2 to RNA (49). In the presence of poly-G RNAs, mdm2 can bind to mutant p53 protein (50), enhancing degradation of mutant p53 and sparing normal p53. This function of mdm2 would tend to bring the cell cycle into control by p53. Each of the aberrant mdm2 products contains solely an intact or partially intact RING domain (Fig. 3, mdm2–281bp, mdm2–254bp, and mdm2–219bp). One possible reason why the breast cancer patients have a remarkably reduced survival is that these aberrant splice products could titrate out factors, such as homopolymeric RNAs, necessary for the binding of mdm2 protein to mutant p53. This would decrease the ability of mdm2 to inhibit mutant p53 proteins, decrease control of the cell cycle, and possibly decrease long-term survival.

Some conclusions can be made on the basis of these observations. First, mdm2 was alternatively spliced in some normal breast samples. Second, mdm2 is both alternatively and aberrantly spliced in some invasive breast carcinomas. Third, these altered mdm2 RT-PCR products were associated with p53 overexpression, suggesting that some p53 overexpression may be related to expression of altered mdm2. Fourth, mdm2 alternative splicing was highly correlated with the presence of p53 mutations, which is suggestive of a mechanism by which mutational inactivation of p53 causes expression of a modified mdm2 protein. This mechanism assumes that p53 mutation occurred before alteration of mdm2. Finally, the presence of aberrantly spliced products is correlated in a statistically significant manner with prognostic markers and with overall patient survival, indicating that the aberrant splicing of mdm2 may have clinical significance. To our knowledge, this is the first time aberrant splicing has been described for mdm2 mRNA and that altered splicing has been correlated with decreased survival in invasive breast cancers. Overall, this work showed that mdm2 could be important in the pathogenesis and outcome of some cases of invasive breast cancer.

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