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Alternative and Aberrant Messenger RNA Splicing of the *mdm2* Oncogene in Invasive Breast Cancer[1]

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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 bp *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 spliced *mdm2* mRNAs and a lack of progesterone receptor. An association was also demonstrated between *mdm2* aberrantly and/or alternatively expression 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 *gadd*45 (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 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 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: (*a*) it inhibits p53 function by binding to the transactivation site of p53 (4); and (*b*) it promotes rapid p53 degradation through a proteasome-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 H_2O, and the absorbance was measured. The RNA was then analyzed for the integrity of the 28S, 18S, and 5S 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-

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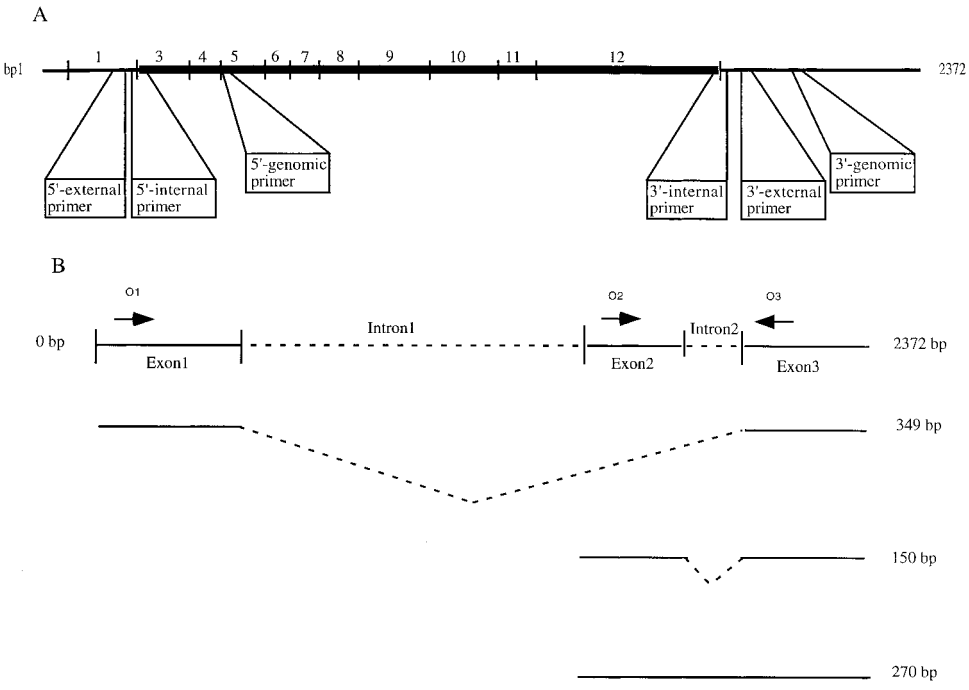

Fig. 1. Location of PCR primers relative to *mdm2* cDNA and genomic DNA structure. *A*, *mdm2* primer sets in relation to the *mdm2* cDNA. The location of nested PCR primers are identified relative to each exon, along with their binding sites on the *mdm2* cDNA sequence. UTRs of the cDNA are denoted by a *thin line*; translated regions of the gene are denoted by a *thick line*. *B*, schematic diagram showing the position of PCR primers 01, 02, and 03 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 P1 promoter activity and two (150 bp and 270 bp) derived from P2 promoter activity. The 350-bp PCR product from P1 promoter activity is known to be p53-independent and lacked exon 2 sequences (presumably attributable to mRNA splicing) but contained the exon 1 sequence (as part of the 5′-UTR) and the exon 3 sequence. The 150-bp PCR product lacked the exon 1 sequence but had the exon 2 and exon 3 sequences. The 270-bp PCR product also lacked the exon 1 sequence but contained the exon 2, intron 2, and exon 3 sequences. This 270-bp product was described by Sigalas *et al.* (16).

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 15-mer oligodeoxythymidylate primer, brought up to a total of 7 μl using RNase-free H_2O, 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), 1× First Strand buffer (Life Technologies, Inc.), 1.0 mM deoxynucleotide triphosphates (Promega), 0.125 mM $MgCl_2$ (Promega), and incubated at 39°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* (1526 bases) was amplified using the following nested primer sets: (*a*) external primer pair: sense, 5′-CTGGGGAGTCTTGAGGGACC-3′, and antisense, 5′-CAGGTTGTCTAAATTCCTAG-3′; and (*b*) internal primer pair: sense, 5′-CGCGAAAACCCCGGGCAGGCAAATGTGCA-3′, and antisense, 5′-CTCTTATAGACAGGTCAACTAG-3′ (Fig. 1*A*). PCR amplification was performed with 25-μl reactions using 1.6 mM $MgCl_2$, 40 pM each primer, 1 mM deoxynucleotide triphosphates, 5 units of 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 with 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′-CCTGTGTGTCGGAAAGATGG 3′) and exon 2 (primer O2-5′-TGTGTTCAGTGGCGATTGGA 3′), respectively, were used with a single 3′ primer located in exon 3 (primer O3-5′ TCTCTTGTTCCGAAGCTGG 3′) as described by Zauberman *et al.* (19; Fig. 1*B*).

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.[4] These primers were: (*a*) sense, 5′-TTCTTTTTTATCTTGGCCAG-3′; and (*b*) antisense, 5′-TCTCATTTAAGACAGAGTAG-3′ (Fig. 1*A*).

***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 or 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 2.1 vector. After transformation, the plasmid was isolated (Wizard plasmid minipreps; 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 in the 5′ end of the antisense primer. 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 cleave 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

[4] The abbreviations used are: *mdm2*, murine double minute 2; UTR, untranslated region; RT-PCR, reverse transcription-PCR; EGFR, epidermal growth factor receptor; ER, estrogen receptor; PR, progesterone receptor; TSG101, tumor susceptibility gene 101; FHIT, fragile histidine triad gene.

detection, as described above, were characterized additionally for specific 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-dideoxyadenosine triphosphate (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 rabbit serum in 31 cases. Nuclear staining was considered positive. In frozen tissue sections, immunostaining is observed in nuclei of a low percentage of normal, proliferatively active tissues. Therefore, we have used 10% immunostained tumor cell nuclei as a value for separation of "normal expression" from "overexpression" (21, 22). Those breast tissues with p53 immunostaining in at least 10% of the cell nuclei were considered to have p53 overexpression, whereas those with <10% p53 immunostained nuclei were considered to be within the normal range of p53 expression.

Immunostaining for the HER2/*neu* oncoprotein was performed on 38 cases as described (23). In brief, rabbit anti-HER2/*neu* was used at a 1:2000 dilution in 10% normal goat serum. HER2/*neu* expression was categorized as described previously (23). Only membrane staining was considered positive.

Thirty-four cases were immunostained for EGFR using the RPN513 antibody (1:25 dilution in 10% normal rabbit serum; Amersham). Tissues were fixed in acetone for 15 min. Because membrane staining is common in normal epithelial cells, only membrane staining in the carcinoma was considered positive.

Thirty-eight cases were immunostained for ER and PR as described previously (24). Immunostaining was described as a percentage of cell nuclei with nuclear staining. For statistical purposes, all nuclear immunostaining was considered positive.

DNA Ploidy Analysis. DNA ploidy analysis was carried out on all 38 cases using the CAS 200 Image Analysis workstation (Cell Analysis Systems), and DNA staining was performed according to the manufacturer's protocol (Cell Analysis Systems, Elmhurst, IL; Ref. 25). In brief, the frozen tissue sample was touched briefly to a prewarmed slide, fixed with 10% formalin, and stained with a modified Fuelgen stain. The amount of DNA in each tumor was evaluated with the CAS 200 system as described elsewhere (25).

Statistical Analyses. The association between *mdm2* expression and the clinical parameters was evaluated using Fisher's exact test and the chi-squared (χ^2) test. The association between *mdm2* expression and overall survival was analyzed using the log-rank test.

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 a 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*).

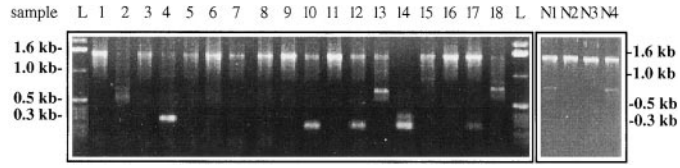

Fig. 2. mdm2 alterations in invasive breast carcinomas and normal breast epithelium. Representative RT-PCR products derived from invasive breast cancers (1–18) and normal breast epithelium (*N1–N4*) were resolved on a 1% agarose gel and visualized with ethidium bromide staining. Full-length *mdm2* cDNA is the predominant transcript in *Lanes 1, 3, 5–9, 11, 15,* and *16*. The 653-bp cDNA predominates in *Lanes 2, 13,* and *18*, whereas smaller transcripts (281, 251, and 219 bp) are noted in *Lanes 4, 10, 12, 14,* and *17*. In *N1-N4*, the full-length *mdm2* transcript predominates over the smaller 653-bp truncated transcript in the two cases where the 653-bp transcript is expressed. The 653-bp transcripts apparently are expressed in normal breast tissues to a much lesser degree than the full-length transcript. All of the illustrated PCR reactions were performed at the same time but in two different reaction sets, one for tumor samples and the other for normal breast samples. *L*, the *Lane*s loaded with the 1-kb DNA ladder (Life Technologies, Inc.). The p53 immunohistochemical staining status and ER/PR status is summarized for each of these breast cancers as follows: *Lane 1*, p53 no sample available, ER+, PR+; *Lane 2*, p53 +, ER+, PR−; *Lane 3*, p53−, ER−, PR−; *Lane 4*, p53 +++, ER−, PR−; *Lane 5*, p53++, ER+, PR+; *Lane 6*, p53 ++, ER+, PR+; *Lane 7*, p53−, ER−, PR+; *Lane 8*, p53−, ER−, PR−; *Lane 9*, p53−, ER+, PR+; *Lane 10*, p53 ++, ER+, PR−; *Lane 11*, p53−, ER−, PR−; *Lane 12*, p53 +++, ER-, PR-; *Lane 13*, p53 ++, ER−, PR−; *Lane 14*, p53 +, ER−, PR−; *Lane 15*, p53−, ER+, PR+; *Lane 16*, p53 ++, ER−, PR−; *Lane 17*, p53 no sample available, ER−, PR−; and *Lane 18*, p53 no sample available, ER−, PR−.

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-bp 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

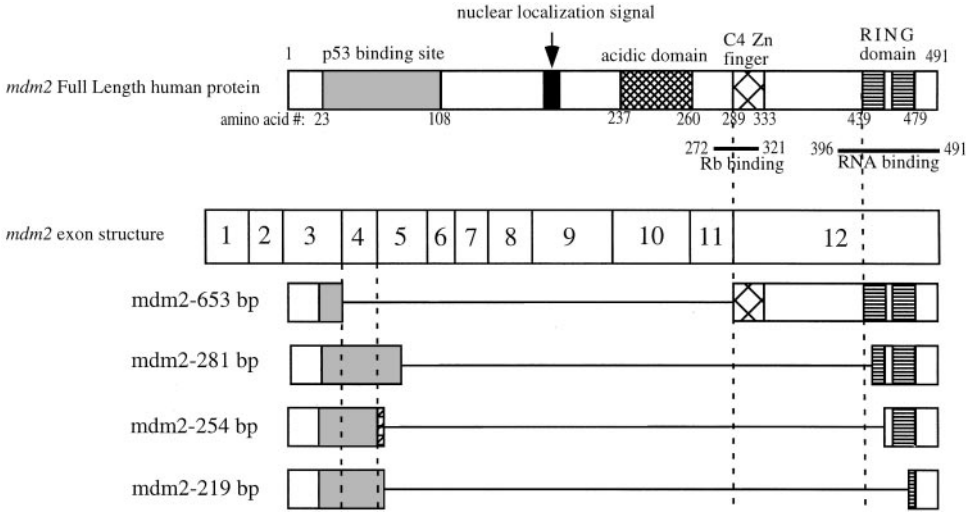

Fig. 3. Schematic diagram of *mdm2* RT-PCR products in relation to the full-length *mdm2* cDNA and functional domains. The *mdm2* full-length cDNA with various protein domain motifs is compared with the exon structure of the mouse *mdm2* gene (26; mouse exon structure), which is 80.3% identical (12) to human *mdm2*, particularly after the start site at base 312. Only the RT-PCR products characterized in this study are illustrated. Identified on each RT-PCR product are the functional domains corresponding to the truncated product. *Dashed lines* have been extended from the mouse exon structure as well as the human domain structure to simplify comparisons. The *gray* domain is the putative p53 binding domain; *black*, the nuclear localization signal; *small crosshatching*, the acidic domain, which binds the L5 ribosomal protein; *large crosshatching*, DNA binding Zn finger domain. The RING finger, a putative RNA binding site, is marked with *horizontal hatching*. The RT-PCR product, *mdm2–254 bp*, has an insertion of 14 bases of DNA, which has homology to intronic sequences and is marked by both *horizontal and diagonal hatching*.

would be in-frame beyond the cryptic splice site. The 281-bp product 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 AGAAGC, and the same sequence was observed at the acceptor site in exon 12 (Fig. 4*A*). Thus the splice donor sequence within exon 5 was 517-*AGAAGC*/AACAAC-524 and the splice acceptor sequence within exon 12 was 1708-*AGAAGC*/TAAAGA-1719

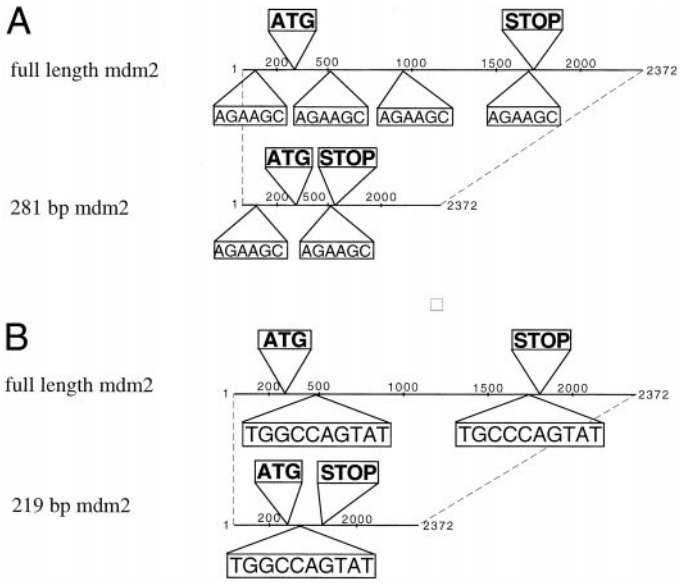

Fig. 4. Comparison of aberrantly spliced *mdm2* RT-PCR products with full-length *mdm2* cDNA. *A*, comparison of the direct repeat sites in full-length *mdm2* (*top*; *full-length mdm2*) with the location of the direct repeat sites identified in the RT-PCR aberrantly spliced 281 bp *mdm2* product (*bottom*; *281 bp mdm2*). The mRNA sequence is represented by a *line* with base numbers; translation start (*ATG*) and stop sites (*STOP*) are indicated *above* the line. The 6-base direct repeat sequences are shown *below* the line. The 281-bp RT-PCR *mdm2* product consisted of 204 bases of 5′ *mdm2* open reading frame sequence followed by the 3′ 76 bases of exon 12 open reading frame retaining only two of the four direct repeat sequences. The location of the presumed donor and acceptor splice sites are described in the text. *B*, comparison of the direct repeat sites in full-length *mdm2* (*top*; *full-length mdm2*) with the location of the direct repeat sites identified in the RT-PCR aberrantly spliced 219-bp *mdm2* product (*bottom*; *219 bp mdm2*). The location of two short direct repeat sequences are indicated in *boxes below the line*. The 219-bp RT-PCR *mdm2* product consisted of 177 bases (exons 3, 4, and the first 16 bases of exon 5) followed by the last 48 bases of exon 12 with retention of only one of the two 10-base repeat sequences in the final product. The putative donor and acceptor splice sites are described in the text.

(italicized 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. 4*A*). A potential cryptic splice site was identified within this repeated 6-bp sequence (AGAAGC). 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, *mdm2–219bp*). The splice site varied around a 10-base site, *TGGCCAGTAT*, in exon 5 and a 10-base site, *TGCCCAGTAT*, 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. 4*B*). 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 intronic 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, the acidic domain, zinc finger 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 δ (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

Table 1 *Summary of p53 mutations in the invasive breast cancers*

No.	Sample	Mismatch detection result	GeneChip mutation	p53 expression
1	C1496	+	Wild type	+++
2	C1506	+	Wild type	ND[a]
3	C1531	+	A138P, R273H	+++
4	C1552	+	S127C, K132N	−
5	C1581	+	Not checked	−
6	C1600	+	Y220C	+
7	C1609	+	C242Y	+++
8	C1620	+	I195F	++
9	C1623	+	P128S, A138P, M160I	+
10	C1626	+	Not checked	ND[a]
11	C1692	+	Not checked	++
12	C1903	+	R267P	ND[a]
13	C1999	+	P36P (polymorphism)	ND[a]
14	C2035	+	L130P, del109, del110	−
15	C2260	+	Wild type	+
16	C2310	+	V157G, G245R	+
17	C2316	+	R213 (polymorphism)	−

[a] ND, not determined.

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 P1 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, P1 and P2, which result in two different 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). The transcript originating from the P1 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. 1*B*) 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. 1*B*). A 350-bp product expected from the splicing of exon 1 to exon 3 without detection of the 150-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 2). 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.

mdm2 alternative and aberrant splicing had statistically significant correlations with clinical parameters. *mdm2* expression was considered to be normal when either a single 1526-bp 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 the 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 2).

If the 1526-bp 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 marginally less significant association ($P = 0.02$) observed when the

Table 2 *Summary of the associations between clinical markers and mdm2 expression*

		mdm2 mRNA expression			
		Considering 1526 bp to be "normal" expression		Considering 1526 and 653 bp to be "normal" expression	
Clinical marker	No. total patients	No. abnormal patients	*P*	No. abnormal patients	*P*
p53 mutation			0.0003		0.02
Negative	29	5		2	
Positive	9	7		4	
p53 IHC[b]			0.023		0.624
Negative	12	2		2	
Positive	19	8		4	
NA[a]	7				
PR			0.036		0.030
Negative	22	11		6	
Positive	16	2		0	
ER			0.087		0.395
Negative	18	9		4	
Positive	20	4		2	
HER2/*neu* oncoprotein			0.228		0.827
Low	24	10		5	
Medium	9	3		1	
High	5	0		0	
EGFR			0.434		0.300
Negative	23	6		2	
Positive	11	5		3	
NA[a]	4				
DNA ploidy			1.000		0.643
Euploid	12	4		1	
Aneuploid	26	9		5	
G_2M phase			0.578		0.115
10%	20	5		2	
>11%	4	2		2	
NA[a]	14				
P1					
Negative	6	2	0.106	1	0.546
Positive	31	9		5	
P2 (150 bp)					
Negative	18	6	1.000	3	1.000
Positive	17	6		3	

[a] NA, tissue not available for analysis.
[b] IHC, immunohistochemistry.

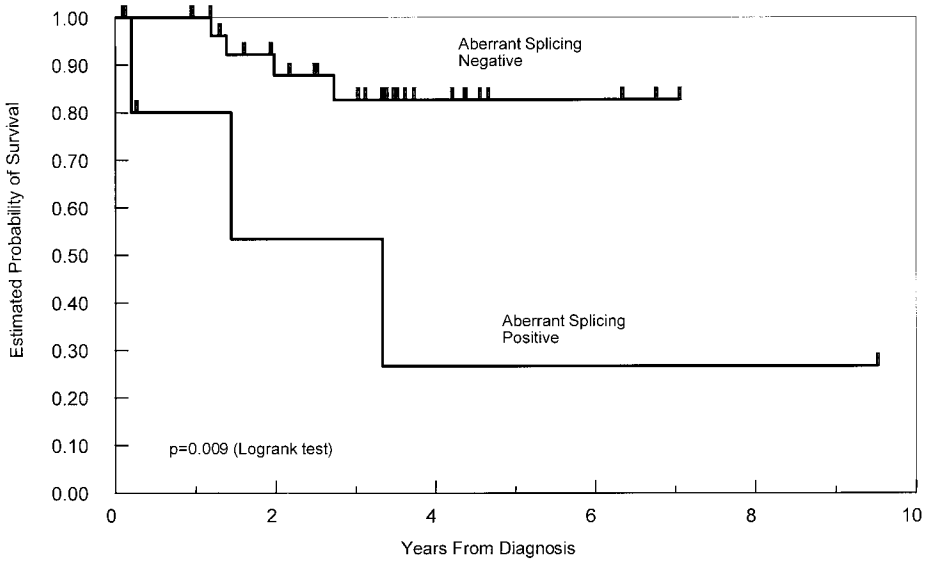

Fig. 5. Log-rank survival curves for breast cancer patients whose breast cancer tissue either contained or lacked aberrantly spliced *mdm2* RT-PCR products. Patients with abnormal *mdm2* RT-PCR products (281-, 254-, and 219-bp products; *solid line*; *Aberrant Splicing Positive*) had a lower overall survival time than did those patients with normal (1526 and 653 bp) *mdm2* RT-PCR products (*hashed solid line*; *Aberrant Splicing Negative*).

653-bp product was considered a normal variant. This change was accounted for, in the main, by the fact that four of 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 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 *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 ($P = 0.0036$; Fig. 5).

DISCUSSION

Relatively little is known about the alternative and aberrant splicing of *mdm2* mRNA. Alternative splicing of *mdm2* transcripts has been described in glioblastomas (28) and astrocytomas (17) as well as in ovarian and bladder carcinomas (16). Five different alternatively spliced *mdm2* transcripts, referred to as *mdm2*-a, *mdm2*-b, *mdm2*-c, *mdm2*-d, and *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 *mdm2*-b. None of the transcripts reported previously were the result of splicing at cryptic splice sites within *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 *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 M_r 57,000 protein (the "normal" size of the mdm2 protein is M_r 90,000) was more highly expressed in neoplastic tissues, which suggested that *mdm2* is altered during progression (29). The present work shows that *mdm2* expression in some breast cancers is characterized by truncated mRNAs <300 bases long. Less than full-length transcripts of *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 *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 *adenomatous 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, *adenomatous 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 *de facto* splice sites. *TSG101* gene and *FHIT* gene mRNAs show evidence of both aberrant and alternative splicing. *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 no discernibly normal splice junction. The *FHIT* gene, another putative tumor sup-

pressor 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 *FHIT* is characterized both by losses of individual exons, loss of exons with insertions of unknown 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 were 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 polyguanylic acid 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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