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FOREWORD

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**ANNUAL REPORT
TABLE OF CONTENTS**

	<u>PAGE NUMBERS</u>
Front Cover.....	<u>1</u>
Standard Form (SF) 298, Report Documentation Form	<u>2</u>
Foreword	<u>3</u>
Table of Contents	<u>4</u>
Introduction.....	<u>5</u>
Body.....	<u>5-8</u>
Key Research Accomplishments	<u>8</u>
Reportable Outcomes	<u>8</u>
Conclusions.....	<u>8</u>
References	<u>9</u>
Appendices.....	<u>10-12</u>

Introduction.

The p53 tumor suppressor gene is important in the pathogenesis of many human cancers including breast cancers. Initially, overexpression of the p53 tumor suppressor protein product was associated with mutations in the p53 gene; however, some cancers lacking mutations in p53 have also been shown to have p53 overexpression by immunohistochemistry. This suggests that other proteins in the p53 regulatory pathway may also be important in the pathogenesis of some cancers. In order to test this hypothesis we evaluated the expression of mdm2 in human breast cancer specimens.

The mdm2 oncogene is regulated by p53 and is responsible for down-regulation of p53 activity through inactivation of p53 function and promotion of p53 degradation. mdm2 inhibits p53 function through binding to the transactivation site of p53. After binding to mdm2 p53 is degraded in proteosomes of the cell. The mdm2 oncogene has been described as amplified in some pediatric sarcomas but not in adult cancers. We evaluated mdm2 gene amplification and expression in a series of 38 frozen breast cancer specimens using Southern hybridization of genomic DNA and reverse transcriptase-PCR of mRNA.

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 μ l reactions, containing 1.6 mM MgCl₂, 40 pM of each primer, 1 mM dNTPs, 5 units of Taq 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 μ l aliquot was transferred directly to a new 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

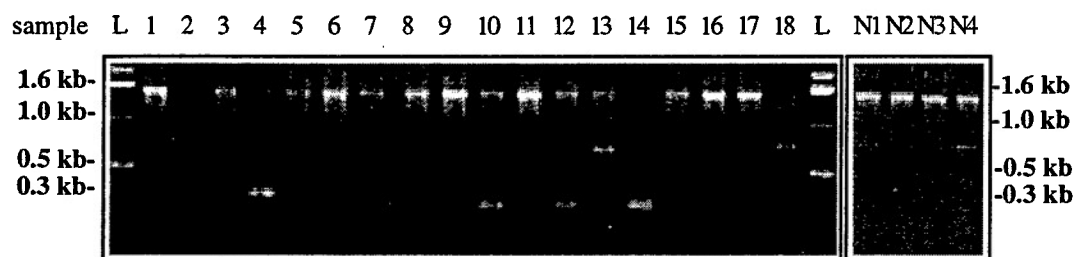


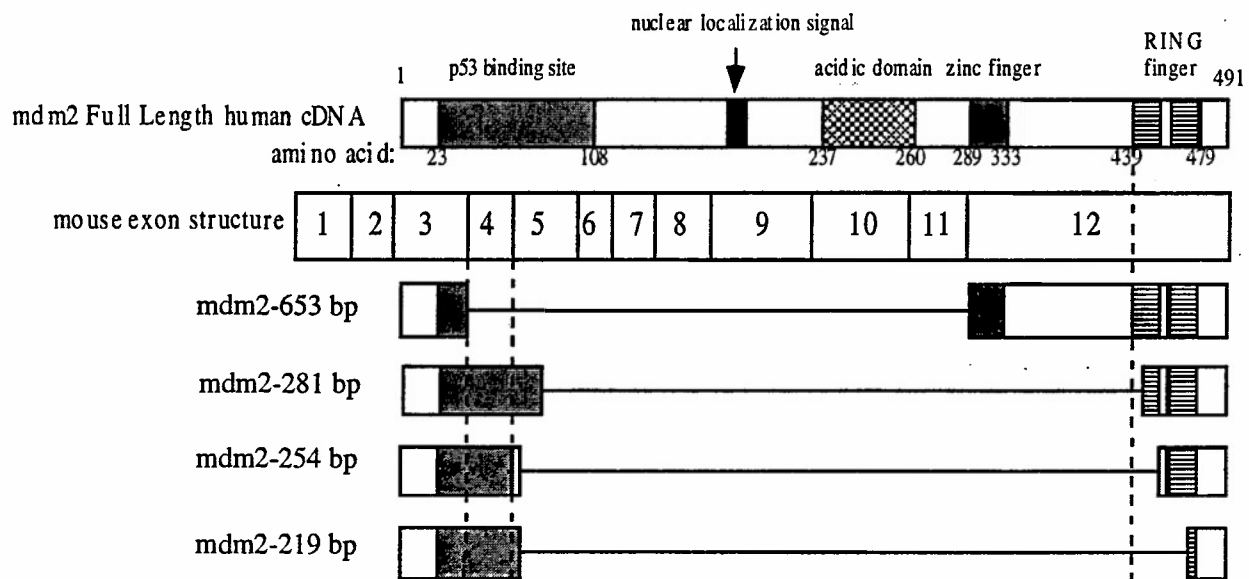
Figure 1. *mdm2* alterations in invasive breast cancers and normal breast tissue. An ethidium bromide stained 1% agarose gel shows RT-PCR products derived from invasive breast cancers (1-18) and normal breast epithelium (N). Samples 1-18 are invasive breast cancer; samples labeled N1-N4 are normal breast 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.

Figure 2. Schematic Comparison of mdm2 with PCR Products of Various Sizes.



A schematic summary is provided comparing the full-length 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. (Canvas 5.0)

The 281 base-pair aberrantly spliced mdm2 product consisted of 204 bases of 5' 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 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 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 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 results in a predicted change in the open reading frame.

In each of these small, aberrantly spliced 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 mdm2 product.

Key Research Accomplishments.

1. Identification of alternative splicing and aberrant splicing of mdm2 in breast cancer.
2. Loss of important functional domain in aberrantly spliced mdm2.

Reportable Outcomes.

None.

Conclusions.

mdm2 mRNA is altered in approximately 30% of human breast cancers. The alteration including loss of functional domains interacting with p53 regulatory domains suggest that mdm2 may play an important role in derangement of the p53 pathway in some breast cancers.

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None.

Appendices.

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