Maintenance of p53 Alterations throughout Breast Cancer Progression

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ABSTRACT

Overexpression of the nuclear phosphoprotein p53 is one of the most common abnormalities in primary human cancer and appears to be due to point mutation within a highly conserved region of the p53 gene which then encodes for a mutant, more stable protein. In this study different stages of breast cancer progression were examined, from in situ to metastatic disease, to determine at what stage mutational activation occurs and whether it is maintained during tumor progression. Two (13%) of 15 pure intraductal tumors expressed high levels of p53 in all malignant epithelial cells. Sequencing of p53 mRNA from one of these tumors demonstrated a nucleotide substitution altering the amino acid composition of the protein. Six (17%) of 35 specimens which contained both in situ and invasive disease expressed high levels of p53. All malignant epithelial cells in these 6 cases were uniformly positive and in no specimen did one component express different levels of the protein than the other growth phase. Sequence analysis of a tissue with significant amounts of both in situ and invasive disease revealed only a single point mutation, without evidence of wild-type nucleotide at the site of substitution, suggesting that p53 mRNA from each component of the tumor contained the same nucleotide substitution. Eleven (50%) of 22 pairs of primary tumors and their lymph node metastases expressed elevated levels of p53, and in each case, expression levels were identical in the primary and secondary sites. Mutations were found in the p53 mRNA from two paired primary and metastatic sites. Therefore, mutation within a highly conserved region of the p53 gene leading to overexpression of the protein product can occur in the earliest recognized phase of breast cancer and this alteration is maintained during progression from intraductal to infiltrating carcinoma. Mutations are also conserved during the process of metastatic spread.

INTRODUCTION

The nuclear phosphoprotein, p53, is found in normal cells but at very low steady-state levels (1). Expression of p53 is tightly regulated throughout the cell cycle (2-4) and may actually be involved in the control of cellular proliferation (5, 6). The exact function of p53 is not fully understood, although recent experiments have shown that the p53 protein can act as a transcriptional activator (7, 8). In addition, the p53 protein is a substrate for cdc2 kinases (9, 10) and can be found complexed to one of these kinases (11), strengthening the relationship of p53 to the regulation of the cell cycle. p53 has been implicated in neoplastic processes because of its ability to transform rodent fibroblasts in cooperation with the ras oncogene (12-14). Only p53 genes that contain mutations in the coding sequence have this dominant transforming capacity (15, 16). In contrast, the murine wild-type p53 gene is able to suppress transformation induced by ras plus mutant p53, E1A, or myc (17, 18). Other evidence has accumulated to suggest that human p53 may also be a tumor suppressor or recessive oncogene (19, 20). A number of different human cancers contain allelic deletions on the short arm of chromosome 17 encompassing the p53 locus and, in many instances, mutations in the remaining copy of the p53 gene have been found, suggesting that loss of the wild-type allele may be a necessary step in the oncogenic activation of p53 in these cancers (21-24).

Mutations in the p53 gene which are oncogenic increase the stability of this normally short-lived protein (25). Therefore, the presence of high steady-state levels of p53 protein can be indicative of an activating mutation. In human breast cancer, we have found that about 25% of primary tumors express elevated levels of the protein and that tumors which have high level overexpression of the protein contain mutations that alter highly conserved amino acid sequences (26). Although these results indicate frequent alteration of p53 in breast cancer and provide a mechanism for activation, the role of p53 in breast cancer initiation and progression remains to be defined.

Carcinoma in situ is the earliest recognizable stage of breast cancer and it is widely assumed that intraductal disease gives rise to invasive cancer. Primary invasive carcinoma of the breast frequently contains both an in situ and infiltrating component; however, the clonal relationship between the two components has not been definitively established. In research performed in our laboratory, equivalent levels of expression of the erbB-2 protein have been found in coexisting in situ and infiltrating components of breast cancer specimens as well as in primary and metastatic lesions from the same patient (27). The maintenance of erbB-2 overexpression suggests that these tumors may have advanced through a clonal progression.

There are fundamental differences in biological potential between the in situ, invasive, and metastatic populations of tumor cells, however. Noninvasive breast cancer is rarely fatal (28), lymph node-negative invasive breast cancer results in at least a 25% mortality, and the mortality from cancer with positive lymph nodes is much higher (29). Therefore, we sought to characterize and compare p53 expression and mutation in these three stages of breast tumor growth.

MATERIALS AND METHODS

Tissue. Human breast cancer specimens from previously untreated patients were collected after surgical removal, immediately flash frozen in liquid nitrogen, and stored at —120°C. Fifteen cases of pure carcinoma in situ (without coexisting invasive disease), 35 cases in which both intraductal and invasive carcinoma could be identified in the same specimen, and 22 pairs of primary infiltrating cancer with a lymph node metastasis were selected for study.

Immunohistochemistry. Eight μm sections of each breast cancer specimen were cut, air dried, and fixed in acetone for 10 min. Immunological detection was performed using the anti-p53 monoclonal antibody, 1801 (Ab-2; Oncogene Science, Manhasset, NY), at 200 ng/ml. This antibody recognizes both wild-type and mutant forms of the p53 protein. Selected specimens were also reacted with an anti-human collagen type IV monoclonal antibody, MAb1 1910 (Chemicon International, Inc., El Segundo, CA), at 100 ng/ml. Binding of these antibodies was visualized using the avidin-biotin-complex (Vector, Burlingame, CA) immunoperoxidase system according to the manufacturer's recommendations. Normal mouse IgG1 (Coulter Immunology, Inc.,

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3 The abbreviations used are: MAb, monoclonal antibody; cDNA, complementary DNA; PCR, polymerase chain reaction.
Hialeah, FL) was used as a negative control. Evaluation of the different patterns of nuclear staining was performed as described previously (26). Only tumors which exhibited intense nuclear staining throughout the malignant epithelium on immunohistochemical analysis with PAb 1801 were categorized as overexpressing p53.

RNA Extraction. Histochemistry slides of each specimen were examined with regard to extent of tumor involvement. Those pieces with significant involvement and minimal lymphocytic infiltration were selected for RNA extraction and subsequent sequencing. RNA was extracted from each specimen using the guanidinium thiocyanate/phenol method (30).

Sequence Analysis. Sequencing of the highly conserved region of the p53 gene from mRNA was performed as previously described (26). Briefly, 1 μg of total RNA was used as a template for p53 first-strand cDNA synthesis by murine leukemia virus reverse transcriptase (BRL, Bethesda, MD) using an antisense oligonucleotide from exon 10 (5'-CTGGGGCATCCTGAGGT-3') as a primer (Duke Comprehensive Cancer Center, Durham, NC). Exons 4 through 10 were then amplified using the PCR (31) by adding an oligo primer from exon 4 (5'-GGGACACGAGCTGCTGACT-3') and Tag DNA polymerase (New England Biolabs, Beverly, MA). The 712-base pair product of this reaction was gel purified, reamplified, and purified again by filtration through a Sepharose CL-6B (Pharmacia, Piscataway, NJ) spin column, ethanol precipitated, and resolubilized in water. This material was the template for dideoxy sequencing using Sequenase 2.0 (United States Biochemical, Cleveland, OH). Oligonucleotides flanking each of the exons 5, 6, 7, and 8 (exon 5: 5'-TACTCCCTCGCCTACAAG-3', 5'-CATCGTATGCGAAGCCT-3'; exon 6: 5'-GTCGGCCTCCCTCCAGAG-3', 5'-CTCAAGGGCGCTCATAGG-3'; exon 7: 5'-CCGAAATCTAGCTTCCGATGTGATGCT-3', 5'-CTGGTGCTCAGTCTGAC-3'; exon 8: 5'-TGTTAATCTACCTGGGA-3', 5'-CTCGTTAGTGCTCC-3') were used to prime the reactions which were performed by first boiling the primer-template mix, labeling on ice for 10 min with [32P]dATP, and then running the termination reactions at 45°C for 10 min. The reaction products were electrophoresed on a polyacrylamide gel which was then soaked in 10% acetic acid/12% methanol, dried, and set with Kodak XAR (Rochester, NY) film overnight.

RESULTS

Expression of p53 in Primary Human Breast Cancers

Carcinoma in Situ. Fifteen specimens were collected in which only intraductal disease could be identified by histopathology. Two (13%) of these tumors had high levels of p53 expression as evidenced by intense nuclear staining in virtually all of the malignant epithelial cells (Fig. 1A). Staining serial sections with an anti-collagen type IV antibody confirmed that the basement membranes were intact around neoplastic ducts in each of these specimens (data not shown). None of the normal epithelial cells had significant staining in any of these tumors and normal murine IgGl controls were uniformly negative (Fig. 1B). Thus, alterations in p53 expression can occur in the earliest morphologically recognizable stage of mammary tumor development but are not present in morphologically normal ductal cells. The homogeneous staining pattern throughout these two intraductal cancers is consistent with the hypothesis that p53 activation is an early event in the progression of some breast cancers.

Coexisting in Situ and Invasive Cancer. A majority of invasive primary breast tumors contain both an in situ and an infiltrating phase of growth. Thirty-five specimens which contained both in situ and invasive disease in the same histological section were reacted with p53 antibody. Six (17%) were found to express high levels of p53 in both components and in virtually all of the malignant epithelial cells in each component (Fig. 1C). Staining with anti-collagen type IV allowed precise delineation of the two components (Fig. 1D). In no instance did one component of growth have high level of protein expression while the other did not. The coordinate expression in these two phases of tumor progression implies maintenance of the underlying genetic alteration during transition from intraductal to infiltrating cancer.

Paired Primary Tumors and Lymph Node Metastases. We next investigated whether altered expression of p53 was acquired or maintained during metastatic progression. Twenty-two pairs of primary tumors and lymph node metastases were collected and all samples underwent immunohistochemical analysis. Eleven (50%) of the primary cancer specimens expressed elevated levels of p53, and in each of these cases, the matched lymph node metastases also overexpressed the p53 protein (Fig. 1, E and G). Staining of serial sections with mouse IgGl served as a negative control (Fig. 1F). Furthermore, in primary tumors containing low or undetectable levels of the p53 protein, no evidence for an increase in expression in a paired metastasis was found. In no case did either the primary or metastatic site alone demonstrate overexpression. Therefore, alterations in p53 expression appear to have occurred prior to metastatic spread and were maintained during this process.

Sequence Analysis of the p53 Message

Carcinoma in Situ. RNA from one of the two specimens of pure intraductal carcinoma containing high level expression of p53 protein was extracted and cDNA was prepared by reverse transcription. From this cDNA, exons 5 through 8 were amplified using PCR and sequenced without cloning. This method minimizes sequencing artifacts due to misincorporations by the polymerase (26). The experimentally determined sequence was compared to wild-type p53 gene sequence determined previously from cDNA from the T-cell line, J6, and genomic DNA from human fetal liver (32). A point mutation in a highly conserved region of the gene (33) within exon 7 was detected in this tumor (Fig. 2). The nucleotide substitution within codon 248 results in the nonconservative amino acid change of glycine to aspartic acid. The wild-type nucleotide and mutant nucleotide were equal in intensity (Fig. 2), suggesting that the wild-type allele may have been retained in this preinvasive lesion. Although tissue blocks were chosen for tumor purity based on histochemical analysis, normal cells admixed in this tumor sample may have contributed some wild-type p53 mRNA. Detection of this mutation confirmed that protein overexpression in in situ disease is associated with mutation in the p53 mRNA, as previously found with primary invasive carcinoma (26).

Invasive Carcinoma with an Intraductal Component. In order to demonstrate that mutational activation of p53 was a clonal event in both in situ and invasive disease, sequence analysis was performed on one of the tumor specimens in which both the intraductal and infiltrating ductal components of the tumor were identifiable and each expressed elevated levels of p53 protein. The tissue was chosen for sequencing because it contained significant amounts of both in situ and invasive disease with few normal cells present. Sequence analysis of the p53 mRNA in this tissue revealed a single point mutation within codon 266 without any evidence of the wild-type nucleotide at the site of substitution (Fig. 3) or any other mutation within the highly conserved regions of the gene implicated in oncogenic activation. The presence of a single point mutation in this tissue in which elevated levels of p53 protein are expressed in both
Fig. 1. Demonstration of p53 overexpression by immunohistochemistry. A, a pure intraductal tumor reacted with the anti-p53 monoclonal antibody, PAb 1801; × 130. B, the same tumor as in A reacted with mouse IgG1; × 130. C, a tumor with both intraductal and invasive disease reacted with PAb 1801; × 180. D, the same tumor as in C reacted with the anti-collagen type IV monoclonal antibody, MAb 1910; × 180. E, a primary, invasive tumor reacted with PAb 1801; × 250. F, the same tissue as in E reacted with mouse IgG1; × 250. G, a lymph node containing metastatic breast cancer in the same patient from whom the primary tumor in E was taken; × 250.
MAINTENANCE OF p53 ALTERATIONS IN BREAST CANCER

A G C T A G C T

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W. T.

In situ

Fig. 2. Detection of a point mutation within the p53 mRNA of an intraductal tumor. Sequence analysis was performed on PCR-amplified p53 cDNA from total RNA extracted from the intraductal tumor shown in Fig. 1A. The autoradiography revealed both the wild-type nucleotide (guanine) and a mutant nucleotide (adenine) within codon 248.

A G C T A G C T

W. T.

Intraductal/Invasive

Fig. 3. Detection of a point mutation within the p53 mRNA of a tumor with both an intraductal and invasive component. Sequence analysis of the tumor shown in Fig. 1C revealed a point mutation within codon 266 in which the wild-type nucleotide, guanine, has been replaced by thymine.

the intraductal and invasive growth phases suggests that both components contained mRNA with the same mutation.

Paired Primary Tumors and Lymph Node Metastases. Two primary tumors and their lymph node metastases, each of which expressed elevated levels of the p53 protein, were sequenced. Point mutations were found in the p53 message from each tumor and each metastatic lesion harbored the same mutation detected in the primary site. This finding suggests that p53 mutations do occur prior to metastatic spread and that these mutations are conserved during this process. Of these two matched sets, one pair appeared to express wild-type p53 also, in both primary and metastasis (Fig. 4), while the other pair appeared to lack wild-type p53 in both (data not shown).

DISCUSSION

Alterations of p53 are among the most common abnormalities detected in primary breast cancer. Levels of p53 expression are normally very low because of its short half-life (1, 25). In established human breast cancer cell lines, however, mutations within the highly conserved regions of the p53 gene result in the expression of a more stable product and, consequently, higher steady-state levels of protein (34). Overexpression of the p53 protein appears to occur in 24–45% of primary breast cancer tissues (35, 36). Tumors which have a high level of p53 protein predictably contain mutations in the coding sequence (26). In contrast, we have detected no mutations in a total of 9 tumors and breast cancer cell lines which demonstrated low or undetectable levels of protein (26). In the current study, different stages of breast cancer progression, from in situ carcinoma to metastatic spread, were examined to determine when overexpression of p53 occurred, whether it was associated with gene mutation, and whether this mutational activation was acquired or maintained during tumor progression. Overexpression of p53 was found in some purely noninvasive breast tumors as well as in metastatic disease. Point mutations were found in all overexpressing tumors which were sequenced, extending the association of mutation with high level expression to all stages of breast cancer.

Primary invasive mammary carcinoma commonly contains both a noninvasive (intraductal) and invasive phase of tumor growth. It is widely held, but not proven, that the noninvasive phase precedes tumor invasion and that one leads to the other. The clonality of these two phases of growth has not been directly demonstrated; however, work in our laboratory has demonstrated maintenance of the level of expression of the erbB-2 oncogene in coexisting components (27). In the current study, six (17%) of 35 cases containing both an infiltrating and intraductal component of growth demonstrated high level p53

W. T.

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Fig. 4. Detection of point mutations within the p53 mRNA of a primary carcinoma and lymph node metastasis. Sequence analysis of the primary tumor shown in Fig. 1E and one of its lymph node metastases (Fig. 1G) revealed that each contained both wild-type (thymine) and mutant nucleotides (adenine) within codon 254.
expression. In each of these tumors, malignant epithelial cells expressed high levels of the protein in both the noninvasive and infiltrating phases of growth. In the remaining 29 cases of coexisting disease, p53 immunoreactivity was absent in both components of growth. One of the six tumors was chosen for extraction of RNA and sequencing because it contained large amounts of intraductal and coexisting invasive disease in close proximity. Only one mutation, a single base substitution in codon 266, was detected in RNA extracted from the mixture of invasive and noninvasive cell populations. Furthermore, the wild-type nucleotide was absent, suggesting that there was deletion of the normal p53 allele in this tumor. Therefore, both the phenotype and genotype of the invasive and noninvasive cell populations with respect to p53 were identical, suggesting that the entire malignant process in this tumor was clonal.

A separate series of 22 paired samples of both the primary tumor tissue and metastatic lymph nodes was examined by immunohistochemistry. There was coordinate expression of p53 in primary and secondary sites of tumor growth with high levels of nuclear staining in tumor tissue from 11 patients. No evidence was uncovered for a change in p53 expression during metastasis. To confirm that these cases contained mutant sequences, RNA was extracted separately from the primary and metastatic tumors in two of these paired samples. In both pairs, identical mutations were found in the primary and secondary sites of tumor growth. These observations confirm the derivation of the metastatic tumor from a clonal population in the primary tumor and are evidence for selection of a mutant p53 gene prior to the metastatic event. One of the metastases sequenced in the current study (removed from a patient with a stage III tumor) appeared to retain both wild-type and mutant alleles. It is possible that the wild-type allele was contributed by RNA from normal lymphocytes or stromal cells in the tumor. However, we have found by in situ hybridization that these elements contribute little p53 mRNA.4 In addition, the equivalent intensity of allelic bands in the sequence analysis of the primary tumor and secondary site implies that either equivalent proportions of normal cells were present in both sites or there was retention of the wild-type allele in both. Retention of the wild-type allele in this tumor and metastasis would suggest that complete elimination of wild-type p53 may not be necessary for a tumor to progress to an advanced stage.

The frequency of p53 overexpression in breast cancer specimens appears to be related to pathological stage; only 2 (15%) of 13 pure intraductal tumors expressed high levels of p53, whereas 11 (50%) of 22 tumors which had invaded regional lymph nodes overexpressed p53. In another, larger study of primary invasive breast cancers, the highest proportion of positively reacting tissues was from patients with stage IV breast cancer (47%) and the lowest fraction (4%) in tissues from patients with stage I disease (37). Yet, in this current study equivalent expression of p53 was found in all paired components (intraductal and invasive, invasive and metastatic), and in every case examined, the mutation present in the more advanced tumors was also present in the less advanced precursors from the same patient. This apparent paradox could be resolved if p53 mutation were an early event in tumor progression which conferred a more aggressive phenotype to tumor cells. These tumors would then rapidly progress through early stages and, therefore, at any time, late stage tumors would have a higher frequency of p53 activations. Under this theory, alterations in p53 may provide prognostic information about the clinical behavior of primary breast tumors. Studies examining the disease-free and overall survival of patients whose tumors express high levels of p53 are currently in progress.

REFERENCES


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