Low Levels of Expression of an Inhibitor of Cyclin-dependent Kinases (CIP1/WAF1) in Primary Breast Carcinomas with p53 Mutations

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ABSTRACT

Recently, several groups have isolated a cell cycle inhibitor gene (CIP1/WAF1) that is highly induced by wild-type, but not mutant forms of the p53 tumor suppressor. To test the hypothesis that p53 regulates CIP1/WAF1 expression in vivo, we evaluated CIP1/WAF1 mRNA expression levels in breast carcinomas from individuals with axillary node-negative disease with and without p53 mutations using quantitative reverse transcription-PCR. The data demonstrate that there is a strong negative correlation between the presence of p53 mutations and CIP1/WAF1 expression, suggesting that p53 mutations may reduce its ability to induce CIP1/WAF1 in vivo. In this study we observed tumors with low levels of CIP1/WAF1 mRNA in which there were no detectable p53 mutations. Determination of the CIP1/WAF1 levels in such specimens may provide a complementary strategy for analyzing the effects of p53 defects and/or may suggest the presence of alterations (such as coding mutations outside the conserved regions, promoter mutations, etc.) that may be missed by standard techniques.

INTRODUCTION

Recently, several groups have independently identified a protein (p21) that has an inhibitory effect on the complexes of cyclins and Cdk's and subsequently on cell cycle arrest (1–3). The p21 protein is encoded by a gene referred to as CIP1 (4), WAF1 (5), SDII (6), or PIC1 (7). Cip1 was initially isolated using a yeast screen for proteins that interact with human Cdk's. Cip1 binds to several cyclin-Cdk complexes and inhibits their kinase activity, hence preventing the complex from driving the cell through the G1-S checkpoint (4). Increases in Cip1 levels result in G1-S arrest (4). Wild-type p53 has also been shown to be involved in growth suppression through cell cycle arrest at the G1-S checkpoint, as seen when p53 levels increase in response to DNA damage (8, 9).

In addition, p53 has been implicated in the regulation of gene transcription (10–13). In fact, WAF1 was identified in a subtractive hybridization system enriched for genes stimulated by wild-type p53 (5). The WAF1 gene has a p53 responsive element located 2.4 kb upstream of the coding region and possesses the ability to suppress growth of human tumor cells.

Until the identification of CIP1/WAF1, and its relationship to p53, there was no clear connection between cell cycle control and genes transcriptionally regulated by p53 (for review, see Ref. 7). However, taken together, the latter observations suggest that CIP1/WAF1 is a mediator of some of the tumor suppressor properties of p53. Consistent with this model, wild-type p53 induces CIP1/WAF1 in cell lines, whereas mutant p53 does not (5). Mutations in p53 may therefore result in a loss or reduction of the transcriptional activation of CIP1/WAF1 and subsequently loss of the tumor suppression functions mediated by CIP1/WAF1.

We have been investigating genetic alterations in primary breast carcinomas in order to understand the etiology of breast cancer. As part of a prospective study to determine the prognostic importance of molecular changes in tumors from women with ANN breast cancer, we have evaluated the frequency and nature of p53 mutations in primary tumor specimens. We show here that 41 of 240 ANN primary breast cancer tumors carry a p53 mutation, and we provide information on the nature of 29 of these alterations. The availability of a large number of breast cancers with and without p53 mutations provided an opportunity to examine the relationship between the status of the p53 gene and the expression of CIP1/WAF1. A quantitative RT-PCR technique was developed to determine the level of expression of CIP1/WAF1 mRNA in the specimens. In this report we show that there is a striking negative correlation between the presence of p53 mutations and mRNA levels of CIP1/WAF1 in primary breast carcinomas.

MATERIALS AND METHODS

Primary Breast Tumor Specimens and Cell Lines. A cooperative system has been established to obtain ANN breast tumors from centers in Toronto (Mount Sinai, Toronto General, Toronto Western, Women’s College, Toronto East General, Saint Joseph’s, North York General, and Credit Valley Hospitals) through collaboration with Dr. D. Sutherland and the steroid receptor laboratory of Sunnybrook Medical Centre. The ANN specimens in this collection are primarily not otherwise specified ductal carcinomas (82.4%) and various histopathological subtypes including lobular (8.2%), mucinous (4%), tubular (2.4%), medullary (2%), papillary (0.6%), and scirrhus (0.4%).
A portion of the primary tumor was quick frozen and stored in liquid nitrogen or at −70°C. Cell lines used in this study were obtained from the American Type Culture Collection. DNA and RNA were extracted using conventional methods (14, 15).

**Multiplex PCR-SSCP.** A modification of the SSCP technique (16) was developed to rapidly analyze large numbers of tumor samples. Exons 5–9 of the p53 gene (the evolutionary conserved region that includes the majority of mutations observed in human tumors) were amplified from tumor DNA with five sets of oligonucleotide primers (17). The PCR amplification of the exons was performed in two separate multiplex reactions: one involving exons 6–8, and the other exons 5 and 9. The radiolabeled multiplex PCR products were analyzed on 6% nondenaturing polyacrylamide gels containing glycerol.4 After autoradiography, variations in the base sequence of the amplified fragments were detected as shifts in electrophoretic mobility. The approximate ratio of p53 mutant alleles: wild-type in tumors was estimated by comparing the intensity of wild-type versus shifted bands on the SSCP gels.

**DNA Sequencing.** Single-stranded template was obtained for DNA sequencing by asymmetric PCR (18) and purified with a Centric 100 microconcentrator (Amicon, Beverly, MA). DNA sequencing was carried out by the dideoxy chain termination method using Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH).

**cDNA Synthesis and Quantitative RT-PCR.** To maximize the usefulness of the tumor RNA specimens a quantitative RT-PCR assay was developed. RT-PCR has the advantage over conventional techniques such as Northern RNA blot analysis in that 10–40-fold less RNA is required. cDNA was prepared from total cellular RNA using random hexadeoxynucleotide primers (Boehringer Mannheim, Postfach, Germany) and Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, MD). cDNA transcribed from 100 ng total RNA was amplified by using Taq DNA polymerase (Perkin Elmer/Cetus, Foster City, CA; Ref. 19). PCR was performed using primer sets for CIP1/WAF1 (5'-AAGACCATGTGGACCTGTCA-3' and 5'-GGCTTCCTC'VFGGAGAAGAT-3'; length of PCR product, 247 bp). Forward and reverse primers were designed specifically to prevent the binding and amplification of pseudogenes or other homologous cDNA sequences. The PCR products were separated by polyacrylamide gel electrophoresis and visualized by ethidium bromide staining, with subsequent quantitation by laser densitometry (Molecular Dynamics, Sunnyvale, CA). Each sample was analyzed at four different PCR cycles (22, 24, 26, and 28 cycles) in separate PCR reactions. Only those reactions which had not plateaued were used in the analysis to ensure quantitative evaluation of the PCR reaction. The majority of the specimens were analyzed at least twice, producing reproducible results.

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**RESULTS**

**Mutations in the p53 Gene.** A total of 240 ANN breast tumors were analyzed by multiplex PCR-SSCP and shifts in electrophoretic mobility (Fig. 1) were observed in 41 (17%) of the specimens. The ratio between mutant and normal alleles varied from 1:2 to 1:9, suggesting that in some cases the mutation occurred in cells which represented a minor component of the tumor. Of the 41 SSCP shifts, 29 were confirmed by direct dideoxy sequencing. The exact p53 mutation could not be accurately determined for specimens which contained predominantly wild-type p53 SSCP fragments because of the limitation of the sequencing technique. Most of the mutations were found to be in exons 5–8. As shown in Table 1, 15 (51%) were nonsense mutations (7 deletions, 3 insertions, 3 stop codons, and 2 splice errors), whereas the remaining 14 were single-nucleotide substitutions (missense mutations) leading to amino acid changes in the p53 protein. Six additional tumors carried the previously reported polymorphism at codon 213. The 12 shifts that have not yet been confirmed by sequencing are not due to the 213 polymorphism (data not shown) as determined by digestion of the PCR products with TaqI restriction enzyme (21).

Of the tumor specimens bearing a p53 mutation on one allele, only four tumors had also lost the wild-type allele. The majority of specimens that contained mutant p53 exhibited a ratio of 1:1 between the mutant and wild-type alleles.

**CIP1/WAF1 mRNA Expression.** Having identified a number of p53 mutant and wild-type breast tumors, we next examined the levels of CIP1/WAF1 mRNA expression in 86 of these samples. Since very low amounts of tumor RNA were available for these specimens, a quantitative RT-PCR assay was developed. Reactions were conducted over a range of cycles (22–24–26–28) to ensure quantitative evaluation within the logarithmic phase of the PCR reaction. To normalize for the amount of RNA and to control for the quality of the RNA samples, each reaction also contained primers for an internal control gene, PGK, for comparison with the level of CIP1/WAF1 mRNA as shown in Fig. 2. As well, the breast cancer cell line MCF-7, which was found to be a relatively high expresser
p53 mutations (irrespective of whether the wild-type allele had p53 expression. Thus, there was a striking negative correlation between the tumors with p53 mutations and the levels of CIP1/WAF1 mRNA.

A few exceptions to this negative correlation were observed (Fig. 4). These included three tumors with p53 mutations in which there were relatively high levels of CIP1/WAF1 mRNA (0.58–0.80 units). However, in all of these cases the mutant to wild-type p53 ratio was low (1.4–1.5), and it seems probable that the final values were distorted by mRNA from cells without p53 mutations in the tumor sample.

An inverse relationship between the level of CIP1/WAF1 mRNA expression and the mutational status of p53 was also detected in several permanent cell lines (Table 2). Four human breast cancer cell lines (MDA-MB-468, T-47D, SK-BR-3, and MDA-MB-231), which previously had been shown to carry p53 mutations (22), exhibited low expression of CIP1/WAF1 mRNA, as did the promyelocytic leukemia cell line HL-60 which bears a large deletion in the p53 gene (22, 23). In contrast, the breast cancer cell line MCF-7 and the human lymphoblastoid cell line RPMI 7666, which only have wild-type p53 alleles, both expressed CIP1/WAF1 mRNA at relatively high levels.

DISCUSSION

There is considerable evidence supporting the involvement of p53 mutations in the development of human neoplasia. In addition to being one of the most common somatic molecular alterations observed in human cancers (24), p53 mutations in the germ line are associated with the predisposition to the development of various cancers in Li-Fraumeni syndrome (25). A number of investigations using immunohistochemical analysis and some molecular analyses have described alterations in p53 in breast cancers (22, 26–35).

In this study mutations in the p53 gene were detected in 17% of 240 ANN breast carcinomas. Using DNA analysis, the percentage of tumors with p53 mutations in several previous studies of primary breast cancers varied from 16 to 33% (22, 26–31). It is of interest that the frequency of p53 mutations observed in this study of ANN breast tumors is similar to that reported for other cohorts which had included cases with positive and negative axillary node involvement.

On the other hand, the frequency of p53 alterations detected by DNA analysis is lower than the frequencies of 23–52% observed in studies using immunohistochemical analysis (31–35). The reasons for the rather extensive variations are not clear. It is possible that DNA analysis that is restricted to the evolutionarily conserved regions of the p53 gene may result in an underestimate of mutation frequency. In addition, the higher frequencies detected by immunohistochemistry could be attributed to some false positives due to different cutoff values set by each set of investigators. As well, proteins which interact with p53 and factors which affect p53 protein stability and expression could also result in a lack of concordance between p53 levels and genetic alterations.

About one-half of all of the p53 mutations found in our analysis of breast tumors involved nonsense-type alterations (microdeletions, microinsertions, stop codons, and splice junction mutations), a value that is much higher than most of the other previous studies. The significance of this observation is not clear. A large percentage (14/19) of single-nucleotide substitutions detected in our study occurred at a G/C base pair, and 10 of those 14 substitutions involved guanosine changes in the noncoding strand, a result which is similar to that observed by Coles et al. (28). This finding supports the notion that guanosine is a preferentially sensitive site and that the DNA damage occurs more commonly on the nontranscribed strand (36, 37).
Relative expression levels of \( CIP1/WAF1 \) (units):

- **MCF-7**: 1.01
- **MDA-MB-231**: 0.46
- **T-47D**: 0.38
- **SK-BR-3**: 0.21

![Quantitation of the levels of \( CIP1/WAF1 \) mRNA expression using 22, 24, 26, and 28 cycles of RT-PCR as described in ‘Materials and Methods.’ The relative expression levels of \( CIP1/WAF1/PGK \) for cell lines MCF-7, MDA-MB 231, T-47D, and SK-BR-3 were determined from the linear region of the reactions to be 1.01, 0.46, 0.38, and 0.21 units, respectively.](image)

![Quantitation of the levels of \( CIP1/WAF1 \) mRNA expression in ANN tumor specimens. The relative expression levels for samples 1532, 1943, 1200, and 851 were determined to be 0.58, 0.80, 0.33, and 0.16 units, respectively.](image)

**Table 2** Evaluation of \( CIP1/WAF1 \) mRNA expression in cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>( p53 ) Status</th>
<th>( CIP1/WAF1 ) level (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>Mutant</td>
<td>0.13</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>Mutant</td>
<td>0.21</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Mutant</td>
<td>0.27</td>
</tr>
<tr>
<td>T-47D</td>
<td>Mutant</td>
<td>0.38</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Mutant</td>
<td>0.46</td>
</tr>
<tr>
<td>RPMI 7666</td>
<td>Wild-type</td>
<td>0.98</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Wild-type</td>
<td>1.01</td>
</tr>
</tbody>
</table>

**Fig. 4** Relative expression of \( CIP1/WAF1 \) mRNA in ANN breast tumors containing wild-type \( p53 \) (■) and mutant \( p53 \) at a 1:1 ratio (□) and less than a 1:4 ratio (▲) of mutant to wild-type \( p53 \).

We observed loss of heterozygosity at the \( p53 \) locus in only 4 of 41 samples with mutations. The observation of wild-type and mutant \( p53 \) in the same specimen may be due to contamination of the samples with nontumor tissue. However, we found that most of the samples with mutations had equal amounts of wild-type and mutant alleles. Although possible, it seems unlikely that these tumor specimens contained an equivalent amount of normal noncancerous tissue. Other investigators have also found retention of the wild-type \( p53 \) allele in primary breast tumors (26, 27, 38). Both wild-type and mutant alleles would be observed if half of the cells in the tumor were homozygous wild-type and the other half were homozygous for the mutant allele or if most of the cells had both a normal and mutant allele. If the latter were the case, it would indicate that inactivation of both alleles of the gene is not always involved in the development of breast cancer.

Cellular heterogeneity within the tumor sample could also represent a confounding factor in the data obtained for \( CIP1/WAF1 \) expression. For example, \( p53 \) mutations may only be present in a minor proportion of the tumor preparation in some samples. Any relationship between such mutations and expression of \( CIP1/WAF1 \) would clearly be mitigated by expression in the tumor cells that do not bear \( p53 \) mutations. Nonetheless, the results provide convincing evidence for an inverse correlation...
between the presence of p53 mutations and the expression of CIP1/WAF1 mRNA in breast tumors.

It is intriguing that such a strong correlation of p53 mutational status and CIP1/WAF1 expression was observed in this study. Although a number of mechanisms for inactivation of p53 have been described it would appear that mutations which affect the p53 gene itself play an important role in determining the level of CIP1/WAF1. On the other hand, we did find a small number of cases with low levels of CIP1/WAF1 mRNA expression in tumors with no detectable p53 mutations. These results could be explained in several ways. There may be p53 mutations in these tumors which we have not detected, either because they are outside the region we examined or because SSCP failed to detect them. Alternatively, mdm2 (39) or other p53 binding proteins which affect p53 function may inhibit p53-mediated transcriptional activation of p53-responsive genes. Finally, there may be other as yet undefined mechanisms by which CIP1/WAF1 expression is regulated.

It was of interest to determine whether our findings were particular to ANN breast cancer or whether they could be extended to breast cancer cell lines and even cell lines of different origins. As shown in Table 2, a very strong correlation between p53 mutations and a decrease in CIP1/WAF1 mRNA expression was detected in the cultured cell lines. The two cell lines, one of which was derived from a breast cancer, which were known to only have the wild-type p53 protein, showed the highest level of CIP1/WAF1 expression. Conversely, all of the cell lines with p53 mutations had CIP1/WAF1 expression in the low range (i.e., below 0.5 units). The HL-60 cell line which carries a deletion in p53 and does not produce p53 protein showed the lowest level of CIP1/WAF1 mRNA expression.

We have analyzed a large group of ANN breast carcinomas and observed the involvement of p53 mutations in 17% of the specimens. It is of clinical importance to determine whether specific molecular alterations are involved in node-negative breast cancer, and it is possible that the level of expression of CIP1/WAF1 mRNA may have the potential to be a prognostic indicator for ANN. Because the CIP1/WAF1 gene product p21 is a likely mediator of many of the effects of p53 in cell cycling and cell growth, we focused on the in vivo effects of p53 mutations on CIP1/WAF1 mRNA expression as a possible mechanism involved in tumor progression. To our knowledge this study is the first report on the relationship between p53 mutations and CIP1/WAF1 gene expression in vivo. These findings strongly suggest that one mechanism by which p53 mutations are involved in carcinogenesis in vivo is through a loss of the ability of p53 to induce CIP1/WAF1. It is possible that analysis of CIP1/WAF1 mRNA levels in tumor samples could be used as an indicator of defects in the upstream regulatory genes of this pathway, in particular the p53 gene. Determination of the CIP1/WAF1 levels in such specimens may provide a complementary strategy for analyzing the effects of p53 defects and/or may suggest the presence of alterations (such as coding mutations outside the conserved regions, promoter mutations, etc.) that may be missed by standard techniques. Future studies of the role of CIP1/WAF1 in carcinogenesis should elucidate the significance of the lower levels of CIP1/WAF1 mRNA.

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