Genetic and Epigenetic Changes in *p21* and *p21B* Do Not Correlate with Resistance to Doxorubicin or Mitomycin and 5-Fluorouracil in Locally Advanced Breast Cancer

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ABSTRACT

Purpose: **The cyclin-dependent kinase inhibitor p21 acts as a main executor of p53-induced growth arrest. Recently, a second transcript, *p21B*, was found to code for a protein expressing proapoptotic activity. We investigated *p21* and *p21B* for mutations and epigenetic silencing in locally advanced breast cancers treated with doxorubicin or 5-fluorouracil/mitomycin and correlated our findings with treatment response and *TP53* status.**

Experimental Design: **We used reverse transcription-PCR to analyze *p21/p21B* mutation status in 73 breast cancer samples. The *p21* promoter region was sequenced and analyzed for hypermethylations by methylation-specific PCR. In addition, a selection of patients were analyzed for mutations in the *p21B* promoter.**

Results: **The *p21* gene was neither mutated nor silenced by promoter hypermethylation in any of the tumors examined. One patient harbored a novel *p21* splice variant in addition to the wild-type transcript. We observed two base substitutions in the *p21* transcript, C93A and G251A, each affecting six patients (8.2%). The G251A variant had not been reported previously. In 12 patients (16.4%), we observed a novel base substitution, T35C, in *p21B*. All three base substitutions were observed in lymphocyte DNA and therefore considered polymorphisms. The polymorphisms did not correlate with p21 staining index, treatment response to doxorubicin or 5-fluorouracil/mitomycin, or *TP53* status.**

Conclusions: **Our findings do not suggest that genetic or epigenetic disturbances in *p21* or *p21B* cause resistance to doxorubicin or mitomycin/5-fluorouracil in breast cancer. Future studies should assess potential associations between these novel polymorphisms and breast cancer risk.**

INTRODUCTION

Although the key factors and functional pathways involved in growth control and apoptosis have been characterized in experimental systems, the interplay between the different factors in response to cytotoxic treatment in human cancers is poorly understood. The tumor suppressor *TP53* plays a pivotal role with respect to apoptosis as well as growth arrest and DNA repair (1–3). Thus, experimental studies have revealed a key role for p53 (the protein coded for by the *TP53* gene) in executing responses to different cytotoxic agents *in vitro* as well as in animal models (4–7). Importantly, we (8) and others (9) have shown that mutations in *TP53* are associated with resistance to anthracyclines, as well as to mitomycin in combination with 5-fluorouracil (10), in breast cancer.

Although *TP53* mutations have been found associated with drug resistance, some observations remain to be explained. Our previous studies revealed a strong association between *TP53* mutations affecting the L2 or L3 loops of the protein and resistance to doxorubicin or mitomycin/5-fluorouracil in primary breast cancer, but some patients expressed chemoresistance despite harboring wild-type (wt) *TP53* (10, 11). This led us to postulate that some of these tumors may suffer damage to other genes involved elsewhere in the "p53 pathway" (12). Recently, we found that a high tumor growth rate, assessed by high mitotic index and Ki67, was associated with a lower response rate in patients harboring wt *TP53* but not those harboring a mutated *TP53* gene (13), suggesting that disturbances in the cell cycle could be an important factor in these patients.

The role of p53 regarding the effects of chemotherapy has been related to its apoptotic function. However, the interplay between p53-induced apoptosis and growth arrest is far from clear (14). The cyclin-dependent kinase inhibitor p21 (the 21-kDa protein encoded by the *p21* gene) is thought to be the main downstream executor of growth arrest triggered by p53 activation (15–18). p21 may bind cdks-2 or -4, generating tertiary complexes with cyclin D or cyclin E (18–27), which interact with several events in cell cycle control.

This study is part of our program evaluating disturbances in different genes involved in the *TP53* pathway, using the same patient materials in which we previously characterized *TP53* alterations in relation to resistance to doxorubicin (11) and mitomycin therapy (10). Recently, a novel transcript from the *p21* gene, designated *p21B*, was reported (28). This transcript partially overlaps the normal *p21* transcript but has a p53 promoter binding site independent from the *p21* promoter region (Fig. 1). It translates into a protein with no sequence overlap

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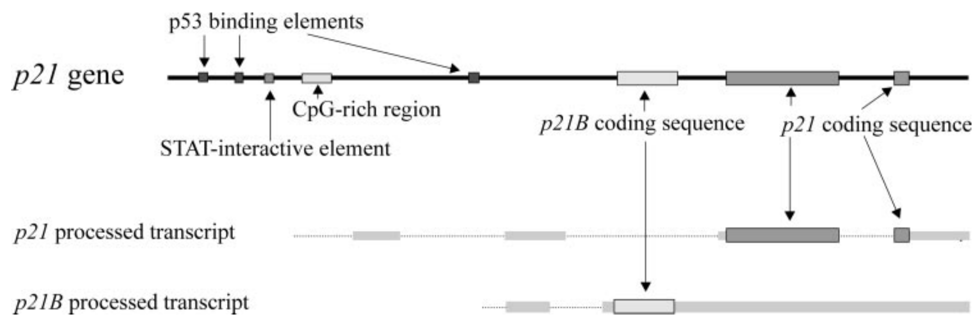

Fig. 1 The *p21* gene. The *p21* gene encodes two proteins, p21 and p21B, with no coding sequence overlap, arising from partially overlapping RNA transcripts. The different transcripts are controlled by distinct promoter regions, both with p53-binding elements.

with p21. Contrary to the p21 protein, p21B has been proposed to regulate apoptosis rather than cell-cycle arrest (28). To our knowledge, *p21B* mutation status as well as promoter methylation of *p21* (*p21B* contains no CpG islands in the promoter region) has not been evaluated in breast tumors previously.

In the present study, we examined mutational status of the coding and noncoding parts of the gene and promoter methylation of *p21* on a selection of tumors from our two previous studies (10, 11). The main hypothesis was to evaluate whether disturbances in p21 function could explain therapy failure to doxorubicin or mitomycin among patients harboring wt p53.

MATERIALS AND METHODS

Patients. This study included patients from two prospective studies addressing the potential role of mutations in *TP53* and other genes regarding resistance to treatment with doxorubicin (11) or mitomycin and 5-fluorouracil (10) in locally advanced breast cancers. Both studies were approved by the Regional Ethical Committee. Because the studies were designed to explore causes of chemoresistance, we focused on comparing tumors that showed primary drug resistance (progressive disease within 12 weeks) with the combined group of those showing stable disease or an objective response (8, 12). Thus, for the patients treated with doxorubicin, we analyzed all tumors that were resistant to therapy together with a randomly selected subgroup of responding tumors (Table 1; Ref. 11). Regarding the group of patients who were treated with mitomycin and 5-fluorouracil, we lacked sufficient material for analysis of one of the tumors previously reported to progress on therapy, but analyzed the remaining 33 tumors (Table 1; Ref. 10). In addition, four patients not participating in these studies were analyzed (Table 1).

RNA Purification. RNA was purified by Trizol (Life Technologies, Inc.) extraction from snap-frozen tissue samples according to the manufacturer's instructions. After extraction, the RNA was dissolved in 100 μl of diethyl pyrocarbonate-treated double-distilled, deionized H_2O, and cDNA was synthesized by reverse transcription-PCR of 4 μl of RNA solution, using Superscript II reverse transcriptase (Invitrogen). The final 20 μl of cDNA solution was not quantified to save material; the amount of cDNA for additional PCRs was determined based on titration of template using cDNA from equivalent tissue samples.

Lymphocyte Cultivation and DNA Purification. Genomic DNA extracted from lymphocytes was available for six patients expressing one or more polymorphisms in *p21/p21B*. In addition, we selected lymphocyte DNA from four patients expressing wt *p21* and *p21B* as controls. Lymphocytes were prepared by cultivating 0.4 ml of blood at 37°C for 3 days in 10 ml of Medium 199 (Life Technologies, Inc.) containing 18% FCS (Life Technologies, Inc.). Genomic DNA was purified from lymphocytes and tumor tissue by use of the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions, followed by sequencing.

PCR Amplification of *p21* and *p21B*. A 607-bp cDNA fragment encompassing the complete *p21* open reading frame was amplified by nested PCR with the primers p21_n2S and p21_n2AS, followed by primers p21_n1S and p21_n1AS (Table 2). Observed mutations were verified by PCR amplification of *p21* exon 2 from genomic DNA, using primers p21_exon2S and p21_exon2AS (Table 2). *p21B* samples were amplified as a 492-bp fragment with use of primers p21B_S1 and p21B_AS1 (Table 2). The *p21B* coding sequence is located in *p21B* exon 2. Because our primers covered a segment of this exon, *p21B* genomic verification of genetic alterations was done with the same primers and PCR program as the cDNA analysis.

Table 1 Characterization of patients investigated in this study by treatment protocol

Treatment	Stable disease/ partial response, *n*	Progressive disease, *n*	Response nonevaluable,[a] *n*
Doxorubicin (11)			
Wild-type p53	60;[b] 12[c]	4[b,c]	
Mutated p53	21;[b] 15[c]	5[b,c]	
5-Fluorouracil and mitomycin (10)			
Wild-type p53	13[b,c]	3[b,c]	1;[b] 3[c,d]
Mutated p53	12[b,c]	6;[b] 5[c]	1[c]

[a] Either nonevaluable for response ($n = 1$) in the original study (10) or not included in that protocol.
[b] Original study population (Ref. 11 for doxorubicin; Ref. 10 for 5-fluorouracil and mitomycin).
[c] Patients examined in the present study.
[d] Including the male patient with the truncated *p21* transcript.

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Table 2 *p21* primers

	Sequence	Position
PCR primers		
p21_nS1	5′-TTCACAGGTGTTTCTGCG-3′	−10 (*p21*)
p21_nAS1	5′-ACACACAAACTGAGACTAAGGC-3′	+557 (*p21*)
p21_nS2	5′-CAGAGCCGAGCCAAGCGTG-3′	−83 (*p21*)
p21_nAS2	5′-GGAACCTCTCATTCAACCGCC-3′	+683 (*p21*)
p21_exon2S	5′-GTAACATAGTGTCTAATCTCCG-3′	Intron 1 (*p21*)
p21_exon2AS	5′-TTTCCTTCTACCTGGAGC-3′	Intron 2 (*p21*)
p21B_S1	5′-GCTGCATGGACTCTGTGATC-3′	−81 (*p21B*)
p21B_AS1	5′-GGCAACTGAAGGGCAAG-3′	+340 (*p21B*)
p21B_proS	5′-TCCATTTCAAACATACCAGTG-3′	−1235 (*p21B*)
p21B_proAS	5′-TGAGTCCTGTGCTGTAGTATTG-3′	−267 (*p21B*)
p21_exon1S	5′-CCAGAGTATTAGGATTACAGGC-3′	−359 (*p21B*)
p21_intron1AS	5′-ATGTCTCTAGGTCTCAGTTTCC-3′	+27 (*p21B*)
MSP[a] primers		
p21_MS1	5′-TACGCGAGGTTTCGGGATCG-3′	−207
p21_MAS1	5′-AAAAACGACCCGCGCTCG-3′	−74
p21_US1	5′-TATGTGAGGTTTTGGGATTGG-3′	−207
p21_UAS1	5′-AAAAACAACCCACACTCAACC-3′	−74
p21_STAT_MS	5′-AGGGTGTAGGGAGATTGG-3′	−780
p21_STAT_MAS	5′-CAAATAAAAAAATTAAAATCCAC-3′	−622

[a] MSP, methylation-specific PCR.

In some tumors, no cDNA *p21B* products were detected after PCR, indicating lack of expression. To verify the integrity of the promoter region in these patients, we amplified fragments spanning positions −1235 to −267 downstream of the p21B start codon, using primers p21B_proS and p21B_proAS, and positions −359 to 27, which are related to the p21B start codon, using primers p21_exon1S and p21_intron1AS, respectively (Table 2).

PCR was carried out with Dynazyme EXT DNA polymerase (Dynazyme) in a 50-μl solution containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM each deoxynucleotide triphosphate, 5% DMSO, 0.2 μM each primer, and 0.5 μl of cDNA or 1 μl of genomic DNA. The *p21* PCR conditions were identical for each nested PCR: an initial 5-min denaturation at 94°C; 30 cycles of 30 s at 94°C, 30 s at 47°C, and 1 min at 72°C; and a final 7-min extension at 72°C. PCR on genomic DNA was done with an initial 5-min denaturation at 94°C; 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C; and a final 7-min extension at 72°C. *p21B* PCR was done with an initial 5-min denaturation at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and a final 7-min extension at 72°C. Finally, the *p21B* promoter PCR was done with an initial 5-min denaturation at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C; and a final 7-min extension at 72°C. After amplification, 15 μl of the PCR products were visualized on a 1% agarose gel.

Cloning of PCR Products. The sample from one patient gave two distinct bands after the *p21* cDNA PCR. Each of the PCR products was cloned into the pCR 2.1-TOPO vector (Invitrogen) for separate analysis. A selection of plasmids was purified by Qiaprep spin mini-prep kit (Qiagen), and the inserts were amplified by M13 PCR (according to instructions provided by Invitrogen) to confirm the insert size.

DNA Sequencing. Sequencing was done directly on 1 μl of the PCR products and plasmids, using Big Dye terminator mixture (Applied Biosystems), with primer sets p21_n1S and p21_n1AS for p21, p21B_S1 and p21B_AS1 for p21B, and p21B_proS, p21B_proAS, p21_exon1S, and p21B_intron1AS for the *p21B* promoter segments (Table 2). After an initial 5-min denaturation at 94°C, the sequencing reaction was carried out for 40 cycles of 10 s at 94°C, followed by 5 s at 55°C and 4 min at 60°C. Twenty plasmids were sequenced to ensure that a possible mutation would be discovered. Capillary gel electrophoresis, data collection, and sequence analysis were performed on an automated DNA sequencer (ABI 3700).

Promoter Methylation Analysis. *p21* promoter methylation status was analyzed by methylation-specific PCR. We modified genomic DNA from patients, using the CpGenome DNA Modification Kit (Intergen), and designed primers specific for methylated (p21_MS1 and p21_MAS1; Table 2) and unmethylated (p21_US1 and p21_UAS1; Table 2) DNA, each PCR product spanning positions −74 through −207 of the p21 promoter sequence. Methylation-specific PCR was done with AmpliTaq Gold DNA Polymerase (Applied Biosystems) in 50 μl of solution containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM each deoxynucleotide triphosphate, 0.2 μM each primer, and 2 μl of modified genomic DNA. The methylated-specific PCR was carried out for 35 cycles of 30 s at 94°C, 30 s at 61°C, and 1 min at 72°C. The unmethylated-specific PCRs were carried out for 35 cycles of 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C. Both PCRs were done with an initial 5 min of denaturation at 94°C and concluded with 7 min at 72°C. After amplification, 18 μl of the PCR products were visualized on a 3% agarose gel.

To complete the methylation analysis of *p21*, we also examined the STAT-promoter binding site at position −692 to the *p21* transcription start site, designated SIE-1 (29). This motif has a single CpG island, analyzed previously by restriction enzyme analysis by Chen *et al.* (29). In 20 samples from randomly selected patients, we amplified a fragment spanning this sequence after DNA modification (as above), using primers p21_STAT_MS and p21_STAT_MAS (Table 2). After an initial

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Table 3 Distribution of *p21* and *p21B* polymorphisms among patients

p21 status	*p21B* status	Number observed
p21 wt[a]	*p21B* wt	53
p21 wt	*p21B* Leu12Pro	8
p21 Ser31Arg	*p21B* wt	3
p21 Ser31Arg	*p21B* Leu12Pro	3[b]
p21 Arg84Gln	*p21B* wt	5
p21 Arg84Gln	*p21B* Leu12Pro	1

[a] wt, wild type.
[b] Test for cosegregation, $P = 0.0521$ (Fisher's exact test).

denaturation for 5 min at 94°C, the PCR was carried out for 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, with a final 7-min extension at 72°C. The PCR products were sequenced directly or cloned into the pCR 2.1-TOPO vector (Invitrogen) and sequenced.

Immunohistochemistry. We previously reported p21 immunostaining for a subgroup of patients belonging to the doxorubicin series as outlined elsewhere (30). In the present study, we compared immunostaining intensity in the subgroup of tumors for which this parameter was available ($n = 35$ of the patients treated with doxorubicin) with promoter hypermethylation, gene mutations, or polymorphism status.

RESULTS

No mutations in the *p21* and *p21B* transcripts were detected. One tumor expressed an alternate splice variant in addition to the *p21* wt transcript. The *p21* variant contained a deletion of bp 47–251 of the open reading frame. The *p21* transcript, which was observed consistently in repeated PCRs, translated correctly the first 16 amino acid residues, followed by a frameshift and further translation of an additional 61 amino acid residues before a stop codon at the end of exon 2. Noteworthy, the tumor harboring this transcript occurred in a male patient and harbored a silent *TP53* polymorphism in position 213 (one of the "nonevaluable" patients; Table 1)

Samples from 12 of 73 patients expressed *p21* polymorphisms (Table 3). The previously reported C93A (Ser31Arg) polymorphism (31–35) was found in six patients (8.2%). In addition, we found a single-base substitution, G251A (Arg84Gln), in six patients (8.2%). To our knowledge, this substitution has not been reported previously. No patients harbored both polymorphisms.

To further confirm that these base substitutions were polymorphic variants, we analyzed the *p21* sequence in genomic DNA from lymphocytes from 10 patients, of whom 4 had the C93A and 2 the G251A base substitutions. For each patient the genomic sequence contained the single-base substitutions observed in the tumor samples. This observation, together with the fact that the G251A substitution was observed in a total of six tumors, strongly suggests that this is a polymorphism, similar to C93A. When we compared our previous results for p21 immunoexpression (30) with the polymorphism status, we observed no difference in the staining scores between those expressing normal p21 and those harboring either the C93A or G251A variants (data not shown). Importantly, *p21* polymorphism status was not related to *TP53* mutation status or response to chemotherapy (data not shown).

Considering the *p21B* transcript, 12 of 73 patients (16.4%) were found to harbor the same single-base substitution, T35C (Leu12Pro; Table 3). Lymphocyte genomic DNA was analyzed for *p21B* alterations in the same 10 patients as for *p21* (above); 3 of these patients harbored concomitant p21B base substitutions in their tumors. The T35C single-base substitution was detected in lymphocyte DNA from all three patients, confirming that the alteration is a polymorphism.

None of the tumors were found to be hypermethylated at the *p21* promoter (data not shown). The *p21B* promoter does not contain CpG islands in relation to the p53 binding site and may not be silenced by methylation.

Eleven tumors did not provide a *p21B* reverse transcription-PCR product and were therefore subjected to genomic DNA analysis. Because the *p21B* promoter does not contain CpG islands, this finding could not be caused by promoter hypermethylation. DNA sequencing confirmed wt gene sequences in the coding domain, introns, and the promoter region. Furthermore, lack of a *p21B* reverse transcription-PCR product was not related to occurrence of the *p21* or *p21B* polymorphisms, nor did it correlate to *TP53* mutation status. Notably, each of the tumors lacking a *p21B* reverse transcription-PCR product expressed normal levels of the *p21* transcript. For those patients harboring a *p21* or a *p21B* base substitution, genomic sequencing revealed equal amounts of the wt and the variant allele; thus, lack of *p21B* expression is not due to large gene deletions, mutations, or loss of heterozygosity.

Interestingly, although we observed no correlation between the $p21^{G251A}$ and $p21B^{T35C}$ polymorphisms, we did find a nonsignificant correlation between expression of the $p21^{C93A}$ and $p21B^{T35C}$ polymorphisms ($P = 0.0521$, Fisher exact test; Table 3). Neither the two p21 polymorphisms nor the $p21B^{T35C}$ polymorphism correlated with *TP53* mutation status or to treatment response.

DISCUSSION

The cyclin kinase inhibitor p21 is thought to be a key executor of p53-induced growth arrest. In addition, it is known to be transcriptionally regulated by factors such as c-Myc and Miz-1 (36, 37), transforming growth factor-β (38), IFN-γ, and STAT1 (39), and it may be regulated at the protein level through direct binding to other proteins, including SET (40) and phosphorylation by Akt (41). Although p21 may bind cdk4 (cyclin D) as well as cdk2 (cyclin E), the evidence at present suggests that it stabilizes the cdk4–cyclin D complex but inhibits the cdk2–cyclin E complex (42, 43), subsequently inhibiting phosphorylation and activation of the retinoblastoma protein in the S-phase (27). Loss of p21 function may lead to activation of the cdk2–cyclin E complex, and recent evidence has linked overexpression of this cyclin, in particular the low molecular forms, to a high risk of relapse in breast cancer (42, 43).

The aim of this study was to explore *p21* status in relation to chemoresistance. Although in previous studies our group (10, 11) found that certain mutations in the *TP53* gene are associated with resistance to doxorubicin and mitomycin treatment in breast cancer, we observed tumors showing chemoresistance

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despite harboring wt *TP53* (44). In the present study, we explored whether disturbances in the *p21* gene could explain some of these findings. Our previous finding (30) that p21 immunostaining did not add predictive information does not exclude a potential role for genetic or epigenetic events in a minor fraction of patients. Although rare (32, 33, 45–53), mutations in the *p21* gene could be of critical importance in some tumors. Furthermore, because a lack of protein staining could be due to several mechanisms, previous findings do not distinguish certain epigenetic events, such as permanent gene silencing through promoter hypermethylation, from other "physiological" mechanisms.

The finding of a truncated *p21* transcript in a tumor harboring wt *p21* is interesting. This patient was not evaluable for response to chemotherapy; thus, we do not know whether this transcript could influence drug sensitivity. To evaluate potential biological functions of this transcript could be an issue for future studies.

We observed two polymorphisms in the *p21* transcript, each occurring in 8.2% of the patients, but no somatic mutations or promoter methylation. The Ser31Arg polymorphism has been linked to cancer susceptibility in one study (54); however, other investigators could not confirm this finding (34, 55–57). Although the G251A (Arg84Gln) base substitution, to our knowledge, has not been reported previously, the fact that it was found in normal tissue from patients harboring the alteration in their tumor strongly suggests that this is a polymorphism.

Recently, a new p53 promoter binding site in the *p21* gene was reported (28). Through this site, p53 induces transcription of a novel p21 variant, named p21B, with a coding sequence with no overlap to p21 (Fig. 1). A recent *in vitro* study suggested a role for p21B with respect to apoptosis (28). We observed an apparent *p21B* polymorphism, Leu12Pro, in 12 of 73 (16.4%) patients, but no somatic mutations. Although reverse transcription-PCR did not reveal a *p21B* mRNA transcript in some of our patients, DNA sequencing and evaluation of promoter integrity excluded *p21B*-specific mutations in these patients, suggesting that this finding could be due to method problems.

Importantly, none of the *p21* and *p21B* polymorphisms were associated with response to therapy, *TP53* status, or p21 immunostaining.

In conclusion, no mutations or promoter hypermethylation in *p21* or *p21B* were identified in any of 73 locally advanced breast cancers. Although we identified two polymorphisms affecting p21 and one polymorphism affecting p21B, none of these polymorphisms correlated with treatment outcome or TP53 status. The fact that two of these polymorphisms, despite being found in 8.2% (G251A) and 16.4% (T35C) of our patients, have not been reported previously raises the question of whether these polymorphisms may be seen among breast cancer patients in particular and thus are associated with disease risk. This hypothesis should be explored in future studies.

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