

The Novel *p21* Polymorphism *p21*^{G251A} Is Associated with Locally Advanced Breast Cancer

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Abstract Purpose: *p21* is a main effector of growth arrest induced by p53. In addition, a second transcript from the same gene (*p21B*) has been linked to apoptosis. We previously analyzed *p21* status in breast cancer and reported two novel polymorphisms of the *p21* gene. In the present study, we present a larger study designed to explore a possible association between these novel polymorphisms and breast cancer.

Experimental Design: The *p21/p21B* polymorphisms were analyzed in 507 breast cancer patients and 1,017 healthy individuals using cDNA or genomic DNA from tumor and/or blood samples.

Results: We detected five polymorphisms of the *p21* gene. Three of these polymorphisms are earlier reported by others, whereas two were reported for the first time in a recent study by us. The presence of the A allele of the *p21*^{G251A} polymorphism was observed more frequently among patients with primary stage III breast cancer (4.5%) compared with stage I and II tumors (1.5%) and healthy female controls (1.4%; *P* = 0.007, comparing the three groups; *P* = 0.0049 and *P* = 0.0057, comparing locally advanced to stage I/II and healthy controls, or to healthy controls alone, respectively). The allele frequencies of the remaining four polymorphisms were evenly distributed among patients and healthy individuals.

Discussion: The finding of an association between locally advanced breast cancer and one particular polymorphism of the *p21* gene suggests this polymorphism to be related to tumor behavior, including enhanced growth rate. If confirmed in other studies, this may add significant information to our understanding of the biology as well as of the clinical behaviour of locally advanced breast cancers.

The cyclin-dependent kinase inhibitor *p21* (*CIP1*, *WAF1*, *CDKN1A*) is thought to be the main executor of p53-induced growth arrest (1–4). Transcriptionally induced by p53, the *p21* protein binds and inhibits the cdk2-cyclin B/E complexes but

increases stability and nuclear localization of cyclin D, stimulating assembly of the cdk4-cyclin D complex (3, 5–13). Interestingly, a second p53-induced transcript from the *p21* gene, *p21B* (Fig. 1) was recently identified and found to encode a protein expressing proapoptotic activity (14), suggesting a role for the *p21* gene in both cell cycle arrest and apoptosis.

We recently described *p21/p21B* status in 73 locally advanced breast cancers (15), observing no promoter methylation or somatic mutations. We did, however, observe two novel polymorphisms, *p21*^{G251A} (Arg84Gln) and *p21B*^{T35C} (Leu12Pro), in addition to the previously known *p21*^{C93A} (Ser31Arg). Although several groups have reported an increased frequency of the *p21*^{C93A} polymorphism in cancer patients (16–20), others reported a similar distribution of the polymorphism in both cancer and healthy individuals (21–25). The frequencies of patients harboring the A allele of *p21*^{G251A} or the C allele of *p21B*^{T35C} were 8.2% and 16.4%, respectively. Although we are not aware of other analyses of the *p21B* coding sequence, the fact that the *p21*^{G251A} polymorphism had not been reported previously made us hypothesize that it may occur at low frequency in non-breast cancer individuals. Analyzing a large set of healthy individuals and breast cancer patients, we here report an increased frequency of the *p21*^{G251A} polymorphism among breast cancer patients. Most interestingly, this

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Grant support: Norwegian Cancer Society and the Haukeland Hospital Program for GeneTherapy.

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doi:10.1158/1078-0432.CCR-05-2822

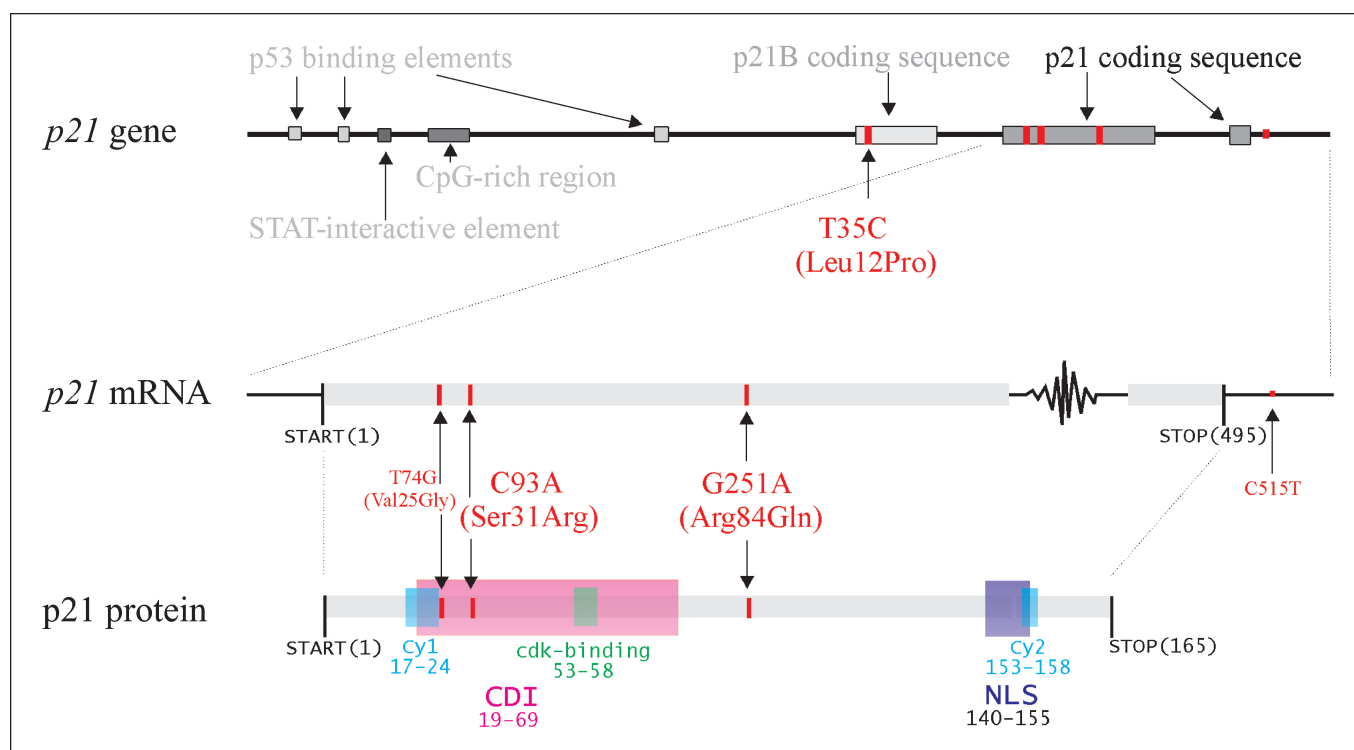


Fig. 1. *p21* and *p21B* with observed polymorphisms. Organization of the coding exons of *p21* and *p21B* with the observed polymorphisms. The functional domains of p21B are not known, but the p21 domains are shown together with the position of the polymorphisms. The CDK inhibitor domain (CDI) is the defining part of the CIP/KIP protein family. p21 contains two cyclin-binding domains (Cy1 and Cy2) and a CDK-binding domain, as well as a nuclear localization domain (NLS). None of the p21 polymorphisms directly affect any functional domains of p21.

increased frequency was limited to the subgroup of breast cancer patients diagnosed with locally advanced tumors.

Materials and Methods

Patients. In this study, we analyzed samples from several cohorts of patients and healthy controls. Ninety-one and 35 patients all suffering from locally advanced breast cancer enrolled in prospective studies exploring resistance to treatment with doxorubicin (26) or mitomycin and 5-fluorouracil (27), respectively, were included. In addition, we analyzed an extra group of six patients with locally advanced breast cancer that were excluded from the mitomycin and 5-fluorouracil study for reasons other than tumor status and 60 patients from a prospective study regarding resistance to paclitaxel. Seventy-three of the locally advanced breast cancers were analyzed in our previous work on p21 (15).

In addition, we analyzed tumor samples from 201 patients suffering from metastatic breast cancer and blood DNA from 170 breast cancer patients (132 stage I-II and 38 stage III) collected at the Norwegian Radiumhospital during the period 1984 to 1990 under an institutional review board-approved protocol.

As controls, we analyzed blood DNA from a total of 1,017 healthy postmenopausal Norwegian females recruited from the national mammographic program into other studies described elsewhere (28, 29).

RNA purification. RNA was purified by Trizol (Life Technologies, Gaithersburg, MD) extraction from snap-frozen tissue samples as per the manufacturer's instructions. After extraction, the RNA was dissolved in 100 μ L DEPC-treated double-distilled water. cDNA was synthesized by reverse transcription-PCR from 4 μ L RNA solution using either Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) or Transcriptor reverse transcriptase (Roche, Indianapolis, IN).

DNA purification. Genomic DNA was prepared from lymphocytes using chloroform/phenol extraction followed by ethanol precipitation

(Nucleic Acid Extractor 340A; Applied Biosystems, Foster City, CA) according to standard procedure for the samples from the Norwegian Radiumhospital, or by QIAamp DNA Mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Before the latter procedure, lymphocytes were prepared by cultivating 0.4 mL blood at 37°C for 3 days in 10 mL Medium 199 (Life Technologies) containing 18% FCS (Life Technologies).

Genotyping of p21 and p21B. The *p21* gene was analyzed on cDNA regarding patients from the doxorubicin, mitomycin and 5-fluorouracil, paclitaxel, and metastatic protocols. Results were verified on genomic DNA from blood when available. The remaining patients and all controls were analyzed on DNA from blood. Amplification of *p21* and *p21B*, both from cDNA and genomic DNA, were carried out as described earlier (15).

One microliter of the PCR product was used as template for sequencing, and the sequencing reaction was done with the Big Dye terminator mix (Applied Biosystems), as described (15). Primers for the sequencing reactions were the same as those used for PCR amplification. Capillary gel electrophoresis, data collection, and sequence analysis were done on an automated DNA sequencer (ABI 3700).

Haplotype analysis. Haplotypes were first constructed using the program PHASE 2.1 (30, 31). PHASE 2.1 was also used to perform permutation testing to determine if there was a difference in the distribution of haplotypes between cases and controls. The permutation test examines the null hypothesis that the case and control haplotypes are a random sample from a single set of haplotype frequencies versus the alternative hypothesis that cases are more similar to other cases than to control haplotypes. The significance was calculated giving the *P* for a permutation test of the null hypothesis. The algorithm was run on 10 permuted data sets for 10 separate times to check for consistency. The final run lasted 10 times longer than the other runs to increase the number of interactions. The final output was the run with the best average value for the goodness of fit variables. The extent of linkage

Table 1. Observations of $p21^{C93A}$

	No. patients analyzed	No. observations			Frequency of individuals carrying the A allele
		Homozygous (CC)	Homozygous (AA)	Heterozygous (CA)	
Healthy controls	1,006	892	1	113	0.113
Stage I/II breast cancers	326	296	2	28	0.092
Locally advanced breast cancers	223	200	1	20	0.094

disequilibrium was expressed as $|D'|$ (recombination rates) and r^2 (association power) as calculated using DNAsp, and significance was calculated using Fisher's exact tests with Bonferroni correction for multiple testing (32). The result was visualized using the GOLD software (33).

The program Haploview (34) was used to perform an independent analysis of the haplotypes in cases versus control, and the result was compared with the results from PHASE analysis. The software was also used to ensure that the genotypes are in Hardy-Weinberg equilibrium, to exclude technical genotyping errors.

Statistical analysis. Each 2×2 test was done using the Fisher's exact test. When three or more groups were compared, analysis was done by the chi-square test. All computations were done using the SAS System (SAS Institute, Inc., Cary, SC); P s reported are two-sided.

Results

In our previous study analyzing locally advanced breast cancers (15), we reported one known ($p21^{C93A}$) and two novel ($p21^{G251A}$ and $p21B^{T35C}$) polymorphisms of the $p21$ gene (Fig. 1). Preliminary screening of the $p21$ and $p21B$ coding sequence in 100 healthy controls (29) indicated that the allele frequencies of the $p21^{C93A}$ and the $p21B^{T35C}$ polymorphisms were equally distributed in normal individuals and tumor samples. However, we found the A allele of the novel $p21^{G251A}$ polymorphism in only 1 out of 100 normal samples compared with 6 of 73 individuals suffering from breast cancer ($P = 0.043$, Fisher's exact test). Based on these preliminary findings, we increased the number of patients and controls to test whether this association would remain statistically significant.

In addition to the three polymorphisms reported in our original study (15), we observed two other $p21$ polymorphisms (Fig. 1). The polymorphism $p21^{C515T}$ is located 20 bp downstream of the $p21$ stop codon and rarely analyzed for but is always reported together with the $p21^{C93A}$ polymorphism (17). Similarly, all patients in our study carrying the $p21^{C93A}$ polymorphism that were analyzed for the region encompassing nucleotide 515 ($n = 27$) also carried the $p21^{C515T}$ polymor-

phism. The G allele of the previously reported $p21^{T74G}$ (Val25Gly) polymorphism (35) was recorded once, in a patient suffering from locally advanced breast cancer (Fig. 1).

Our observations on $p21^{C93A}$, $p21^{G251A}$, and $p21B^{T35C}$ are summarized in Tables 1, 2 and 3 respectively. Homozygosity for the rare allele is infrequent (Tables 1-3), and all further statistical analyses thus concern wild-type individuals versus the combined groups of heterozygous and homozygous for the rare allele. The $p21^{C93A}$ and $p21B^{T35C}$ polymorphisms occur at similar frequencies among healthy controls and cancer patients. (Frequency of presence of the rare allele was 9-11% for each polymorphism in each group; Tables 1 and 3). $p21^{G251A}$ is more infrequent, present in only in 1.9% of the samples (Table 2). Although presence of the rare allele is observed more frequently in patients (2.7%) than in controls (1.0 %), the difference is not statistically significant ($P = 0.076$, Fisher's exact test). Dividing our patients into two subgroups based on tumor stage, we found the $p21^{G251A}$ variant in 4.5% of our locally advanced breast cancers versus 1.5% among individuals diagnosed with stage I and II tumors ($P = 0.0526$, Fisher's exact test). Comparing the frequency of $p21^{G251A}$ among healthy controls, stage I/II patients, and locally advanced patients, a clear difference between the three groups was detected ($P = 0.007$, χ^2). Similarly, comparing stage III cancer patients with either stage I/II patients and healthy controls together, or healthy controls alone, we found a statistically higher frequency of carriers of the A allele of the $p21^{G251A}$ polymorphism among stage III patients ($P = 0.0049$ and $P = 0.0057$, respectively, Fisher's exact test).

The haplotype structure, considering all the five coding polymorphism in the $p21$ gene was determined. Overall, six different haplotypes were observed for the combined cases and controls data set. PHASE calculated six different haplotypes for the case group and four for the controls. Two infrequent haplotypes were found in the cases only, whereas no control-specific haplotypes were observed. A case-control permutation test did not show a significantly different distribution of haplotypes in cases compared with controls ($P = 0.47$).

Table 2. Observations of $p21^{G251A}$

	No. patients analyzed	No. observations			Frequency of individuals carrying the A allele
		Homozygous (GG)	Homozygous (AA)	Heterozygous (GA)	
Healthy controls	1,006	996	1	13	0.014
Stage I/II breast cancers	326	321	0	5	0.015
Locally advanced breast cancers	223	213	0	10	0.045

Table 3. Observations of *p21B*^{T35C}

	No. patients analyzed	No. observations			Frequency of individuals carrying the C allele
		Homozygous (TT)	Homozygous (CC)	Heterozygous (TC)	
Healthy controls	1,017	917	3	97	0.098
Stage I/II breast cancers	333	299	0	34	0.102
Locally advanced breast cancers	219	191	0	28	0.128

Discussion

In this article, we report the presence of previously identified as well as novel polymorphisms of *p21/p21B* among 474 Norwegian breast cancer patients and 1,017 healthy individuals.

The polymorphisms are rarely found together (with the exception of the linkage between *p21*^{C93A} and *p21*^{C515T}), and when two are observed together, they are always heterozygous. This indicates that no haplotype containing more than one of the polymorphism *p21*^{T74G}, *p21*^{G251A}, or *p21B*^{T35C}; or both *p21*^{C93A} and *p21*^{C515T} are present among patients and controls analyzed. This constitutes five of the six PHASE reported haplotypes (including the haplotype wild type in all positions). The final haplotype predicted by PHASE is disregarded, as it results from estimation of status for one of the polymorphisms, and thus may not be counted among our observed haplotypes. We here refer to the haplotypes as wild type or by the polymorphism defining the haplotype, as no haplotype carries two or more of the *p21*^{T74G}, *p21*^{G251A}, *p21B*^{T35C}, or *p21*^{C93A} polymorphisms.

Expanding on our previous results (15), we observe the novel *p21* polymorphism, *p21B*^{T35C}, as well as the previously defined *p21*^{C93A}, to occur with a similar frequency among breast cancer patients and non-breast cancer individuals. Although the *p21*^{G251A} polymorphism was observed more frequently among breast cancer patients compared with controls, this difference did not reach statistical significance. In contrast, we found this polymorphism to be significantly associated with stage III (locally advanced) breast cancer. Although locally advanced breast cancer seems to present cDNA microarray profiles resembling the profile of breast tumors in general (36, 37), recent studies suggest a potential difference in biology between tumors detected by mammographic screening and so-called "interval cancers" (38). "Interval cancers" are thought to include subgroups of fast-growing tumors. Many of our patients revealing a large tumor at time of diagnosis had recognized symptoms for short time intervals only (26, 27), and the possibility exists that some locally advanced cancers may have fast-growing tumors due to particular genetic disturbances. Considering *p21* to be critical to growth arrest, any disturbance in the function of this gene is expected to affect cellular growth

rate, explaining an association with locally advanced tumor development. Ongoing studies in our laboratory are exploring the function of the different alleles of this polymorphism in the *p21* protein's ability to inhibit cellular growth.

When observing a novel polymorphism of *p21* the possibility exists that this is a geographically limited variant. However, linking *p21*^{G251A} to the subset of locally advanced breast cancers presents another plausible explanation as to why the variant has not been reported previously. Ours was the first study addressing the full *p21* status of locally advanced breast cancers, whereas many earlier screenings only evaluated status of *p21* polymorphisms already known. As the frequencies of the polymorphism in stage I/II tumors and among healthy individuals are low, a geographic limitation of the variant is not necessary to explain that others did not observe the *p21*^{G251A} polymorphism.

As the *p21*^{G251A} polymorphism is rare, homozygotes are expected to be very low in number. We observe only one homozygote in all our samples, and we are thus not able to evaluate the significance of homozygosity versus heterozygosity in relation to the clinical implications of this polymorphism.

Although the *p21B*^{T35C} polymorphism has previously been analyzed in only one small case control study and not investigated in larger cohort studies, conflicting evidence has linked the *p21*^{C93A} polymorphism to cancer risk as well as prognosis (16–25, 39). We found both polymorphisms to be evenly distributed among our groups of individuals, showing no association to any clinical aspect of our patients.

Currently, there is much focus on "low-risk" genes with respect to breast cancer risk (40). If the association of the *p21*^{G251A} polymorphism to locally advanced, but not other forms of breast cancer, is confirmed, it suggests we may start looking not at elevated risk of breast cancer in general, but rather whether particular tumor forms may reveal a family association. Notably, whereas tumors in *BRCA2* carriers in general reveal a biology resembling the biology of tumors in patients with no germ line mutation, the opposite is the case for tumors among *BRCA1* carriers (41–43).

In conclusion, we find the novel *p21*^{G251A} polymorphism to be associated not to breast cancers in general but to a subgroup of the disease characterized by aggressive growth.

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