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, 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 (GIP1, 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 p21\(^{T35C}\) (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 p21\(^{T35C}\) 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...
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 µL DEPC-treated double-distilled water. cDNA was synthesized by reverse transcription-PCR from 4 µL 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
frequencies of the sequence in 100 healthy controls (29) indicated that the allele was equally distributed in normal individuals and tumor samples. However, we found the A allele of the novel polymorphism that were analyzed for the region encompassing nucleotide 515 (Val25Gly) polymorphism (35) was recorded once, in a patient suffering from locally advanced breast cancer (Fig. 1).

Our observations on p21C93A, p21G251A, and p21B74C 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 p21C93A and p21B74C 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). p21G251A 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 p21G251A 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 p21G251A among healthy controls, stage I/II patients, and locally advanced patients, a clear difference between the three groups was detected (P = 0.007, \( \chi^2 \)). 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 p21G251A 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 1. Observations of p21C93A

|                      | 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            |

### Table 2. Observations of p21G251A

|                      | 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            |

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 p21C93A and p21G251A), and when two are observed together, they are always heterozygous. This indicates that no haplotype containing more than one of the polymorphism, p21T74G, p21G251A, p21B T35C, or p21C93A and p21G251A, 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 more than one of the p21 polymorphisms.

Expanding on our previous results (15), we observe the novel p21 polymorphism, p21T35C, as well as the previously defined p21C93A, to occur with a similar frequency among breast cancer patients and non-breast cancer individuals. Although the p21G251A 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 breast cancers among patients and non-breast cancer individuals. Although the p21C93A and p21G251A 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 p21G251A polymorphism.

As the p21G251A 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 p21B735C polymorphism has previously been analyzed in only one small case control study and not investigated in larger cohort studies, conflicting evidence has linked the p21C93A 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 p21G251A 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 p21G251A polymorphism to be associated not to breast cancers in general but to a subgroup of the disease characterized by aggressive growth.

References

1. Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ. Radiation-induced cell-cycle arrest compromised by p21 deficiency. Nature 1995; 377:552–7.
2. Deng CX, Zhang PM, Harper JW, Elledge SJ, Leder P. Mice lacking p21(C)/p19(Ink4a) undergo normal development, but are defective in G1 checkpoint control. Cell 1995;82:675–84.
3. Dulic V, Kaufmann WK, Wilson SJ, et al. P53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell 1994;76:1013–23.
4. Luo Y, Hurwitz J, Massague J. Cell-cycle inhibition by independent Cdk and Pcn binding domains in P21(Cip1). Nature 1995;376:159–61.
5. Chong MG, Olivier P, Dehl JA, et al. The p21(Cip1) and p27(Kip1) CDK ‘inhibitors’ are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J 1999;18:1571–83.
6. Dulic V, Stein GH, Far DF, Reed SI. Nuclear accumulation of p21(Cip1) the onset of mitosis: a role at the G(2)/M-phase transition. Mol Cell Biol 1998;18: 546–57.
7. Gu Y, Turk CW, Morgan DO. Inhibition of Cdk2

Table 3. Observations of p21B<T35C>

| No. patients analyzed | No. observations | Frequency of individuals carrying the C allele |
|-----------------------|-----------------|---------------------------------------------|
| Healthy controls      |                 |                                             |
| 1,017                 | 917             | 0.098                                       |
| Stage I/II breast cancers | 333             | 0.102                                       |
| Locally advanced breast cancers | 219            | 0.128                                       |
|                       | Homozygous (TT) |                                |
|                       | Homozygous (CC) |                                |
|                       | Heterozygous (TC) |                            |
| 3                     | 0               |                                             |
| 97                    | 34              |                                             |
| 28                    | 0               |                                             |
activity in vivo by an associated 20k regulatory subunit. Nature 1993;366:707–10.
8. Harper JW, Adami GR, We N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein kinase is a potential inhibitor of G1 cyclin-dependent kinases. Cell 1993;75:805–16.
9. Medema RH, Klompmaker R, Smits VAJ, Rijksen G. p21 (waf1) can block cells at two points in the cell cycle, but does not interfere with processive DNA-replication or stress-activated kinases. Oncogene 1998;16:431–41.
10. Niculescu AB, Chen XB, Sneets M, Hengst L, Prives C, Reed SI. Effects of p21 (Cip1/Waf1) at both the G1(S) and the G(2)/M cell cycle transitions: pRb is a determinant in blocking DNA replication and in preventing endoreduplication. Mol Cell Biol 1998;18:629–43.
11. Sherr CJ, Roberts JM. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 1995;9:1149–63.
12. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999;13:1501–12.
13. Xiong Y, Hannon GJ, Zhang H, Cedo D, Kobayashi R, Beach D. P21 is a universal inhibitor of cyclin kinases. Nature 1993;366:701–4.
14. Nozol S, Chen X. p21B, a variant of p21 (Waf1/Cip1), is induced by the p53 family. Oncogene 2002;21:1285–94.
15. Staalesen V, Leirvaag B, Lillegaard JR, Lonning PE. 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. Clin Cancer Res 2004;10:3438–43.
16. Wen WC, Wu HC, Hsu CD, Chen HY, Tsai FJ. p21 gene codon 31 polymorphism is associated with bladder cancer. Urol Oncol 2002;7:63–6.
17. Facher EA, Becich MJ, Deka A, Law JC. Association between human cancer and two polymorphisms occurring together in the p21(WAF1/Cip1) cyclin-dependent kinase inhibitor gene. Cancer 1997;79:2424–9.
18. Harima Y, Sawada S, Nagata K, Sougawa M, O斯塔penko V, Giroshi T. Polymorphism of the WAF1 gene is related to susceptibility to cervical cancer in Japanese women. Int J Mol Med 2001;7:261–6.
19. Roh J, Kim M, Kim J, et al. Polymorphisms in codon 31 of p21 and cervical cancer susceptibility in Korean women. Cancer Lett 2001;58:59–62.
20. Sjolander A, Birgander R, Rannug A, Alexandrie AK, Tomling G, Beckman G. Association between the p21 codon 31A (arg) allele and lung cancer. Hum Genet 1996;96:221–5.
21. Hsieh YY, Tsai FJ, Chang CC, et al. p21 gene codon 31 arginine/serine polymorphism: non-association with endometriosis. J Clin Lab Anal 2001;15:184–7.
22. Resnissiotis T, Griffiths PG, Keers SM, Chinnery PF. p21B, a polymorphism at codon 31 of gene p21 is not associated with primary open angle glaucoma in Caucasians. BMC Ophthalmol 2005;5:9.
23. Shih CM, Lin PT, Wang HC, Huang WC, Wang YC. Lack of evidence of association of p21(WAF1/Cip1) polymorphism with lung cancer susceptibility and prognosis in Taiwan. Jpn J Cancer Res 2000;91:9–15.
24. Su L, Liu G, Zhou W, et al. No association between the p21 codon 31 serine-arginine polymorphism and lung cancer risk. Cancer Epidemiol Biomarkers Prev 2003;12:174–7.
25. Bai MH, Chen WC, Tsai FJ. Correlation of p21 gene codon 31 polymorphism and TNF-alpha gene polymorphism with nasopharyngeal carcinoma. J Clin Lab Anal 2002;16:146–50.
26. Geisler S, Lonning PE, Aas T, et al. Influence of TP53 gene alterations and c-erbB-2 expression on the response to treatment with doxorubicin in locally advanced breast cancer. Cancer Res 2001;61:2505–12.
27. Geisler S, Berresen-Dale A-L, Johnsen H, et al. TP53 gene mutations predict the response to neoadjuvant treatment with FUMI in locally advanced breast cancer. Cancer Res 2003;63:5582–8.
28. Gram IT, Bremnes RD, Ursin G, Maskarinec G, Sjalander A, Birgander R, Rannug A, Alexandrie AK, Johnsen H, et al. TP53 and breast cancer. Proc Natl Acad Sci USA 2002;99:10869–74.
29. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2000;97:10058–65.
30. Rebbeck TR, Lynch HT, Neuhausen SL, et al. Prophylactic oophorectomy in carriers of BRCA1 or BRCA2 mutations. N Engl J Med 2002;346:1616–22.