

SHORT REPORT

Mutations and polymorphisms of the *p21B* transcript in breast cancer

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p21^{WAF1/CIP1}, transcribed from the *CDKN1A* locus, plays a key role executing p53-induced growth arrest. The recent discovery that an alternative transcript, *p21B*, induces apoptosis, suggests an additional important function of this gene. Here, we report *p21* and *p21B* mutation status in large cohorts of breast cancers and compare distributions of *p21B* polymorphisms in cancer patients to healthy controls. In 521 breast tumor samples analyzed, only one point mutation affecting the *p21B* protein was observed. No mutations were found when screening a panel of 20 established cell lines. A novel polymorphism, *p21B*^{G128T} was identified. Haplotype analysis revealed no association between this variant and the previously identified *p21B* polymorphism *p21B*^{T35C} or any of the known *p21*^{WAF1/CIP1} polymorphisms. As previously reported for *p21B*^{T35C}, distribution of *p21B*^{G128T} was similar among breast cancer patients and healthy controls ($n = 691$ and $1,015$; incidence 6.1 vs. 4.8% ; $p = 0.273$, respectively). No association between *p21B*^{G128T} or *p21B*^{T35C} and response to chemotherapy with an anthracycline-containing regimen or paclitaxel was recorded. Our findings do not suggest mutations or polymorphisms of *p21B* to play a major role with respect to either breast cancer risk or sensitivity towards chemotherapy.

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p21B is a recently discovered protein translated from an alternative transcript of the *CDKN1A* gene, commonly known as the locus encoding *p21*^{WAF1/CIP1}. Expression of *p21B* is driven by an independent promoter, and the processed transcript consists of a short first exon spliced to a longer exon harboring the *p21B* coding region. The promoter as well as the complete coding region of *p21B* is located within intron 1 of *p21*^{WAF1/CIP1}, thus, the two proteins do not share any amino acid sequence.¹

Transcription of *p21B*, similar to transcription of *p21*^{WAF1/CIP1}, is induced by p53.¹ However, while *p21*^{WAF1/CIP1} is a mediator of cell cycle arrest through inhibition of the cyclin E-CDK2 complex, *p21B* has been linked to induction of apoptosis.¹ As a few studies have examined somatic mutations affecting *p21*^{WAF1/CIP1} in human cancers,^{2–7} no study has examined mutations affecting the *p21B* transcript. Several polymorphisms within the *CDKN1A* locus have been reported, the most prevalent being *p21*^{C93A}. Case control studies on this variant has provided conflicting evidence, suggesting an association to risk of different cancer forms.^{8–16} In contrast, the only *p21B* polymorphism previously identified is *p21B*^{T35C},¹⁷ previously reported by us not to be associated with breast cancer risk.¹⁸

We here present the results from screening of 521 breast tumor samples for somatic alterations in the *p21*^{WAF1/CIP1} and *p21B* transcripts. Further, we screened blood samples from an additional 170 breast cancer patients (132 patients with operable stage I and II breast cancers and 38 patients with stage III disease) together with samples from 1,015 healthy individuals for *p21B* polymorphisms.

Material and methods

Patients

In the first part of this study, we examined tumor tissue from a total of 320 primary locally advanced breast tumors for potential somatic alterations in the *p21B* transcript. These patients participated in neoadjuvant chemotherapy trials described elsewhere,^{19,20} or ongoing studies approved by the regional ethical committee. Considering that genetic alterations could be different in primary and metastatic tissue, we also analyzed tumor samples from 201 patients suffering from metastatic breast cancer enrolled in an ongoing study approved by the regional ethical committee.

Recently, we discovered a novel *p21* polymorphism that was not associated with breast cancer risk in general but was particularly frequent among patients harboring locally advanced tumors. On the basis of identification of a novel *p21B* polymorphisms, *p21B*^{G128T}, in cancer patients in the first part of the study, we aimed at exploring its distribution in cancer patients *versus* controls, in the second part of the study. Thus, we included blood samples from an additional 170 cancer patients (132 stage I/II breast cancer patients and 38 patients with locally advanced breast cancer) as well as blood samples from 1,015 postmenopausal Norwegian women, with a negative screening mammogram, described elsewhere.^{21,22}

RNA purification

Total RNA was isolated from snap-frozen tissue samples using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. RNA was subsequently dissolved in DEPC-treated double-distilled water. Single strand cDNA was synthesized from total RNA using either Superscript II reverse transcriptase (Invitrogen, San Diego, CA) or Transcriptor reverse transcriptase (Roche, Indianapolis, IN) according to the manufacturer's instructions.

DNA purification

Genomic DNA was purified using QIAamp DNA Mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions, or by phenol/chloroform extraction followed by ethanol precipitation (Nucleic Acid Extractor 340A; Applied Biosystems, Foster city, CA).

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TABLE I – CELL LINES INVESTIGATED FOR *p21B* MUTATIONAL STATUS

Cell line	Tissue of origin	<i>p21B</i> status
A431	Epidermoid carcinoma	Wildtype
NB4	Promyelocytic leukemia	Wildtype
HeLa	Cervical adenocarcinoma	Wildtype
NCI-H1299	Nonsmall cell lung cancer	Wildtype
GaMg	Glioma	Wildtype
MCF-7	Mammary gland adenocarcinoma	Wildtype
AU565	Mammary gland adenocarcinoma	Wildtype
MDA-MD 468	Mammary gland adenocarcinoma	Wildtype
MDA-MD 2310	Mammary gland adenocarcinoma	Wildtype
HCC 1569	Mammary gland adenocarcinoma	Wildtype
HCC 1395	Mammary gland ductal carcinoma	Wildtype
MCF-10A	Nontumor mammary gland epithelium	Wildtype
HCT 116	Colorectal carcinoma	Wildtype
SW48	Colorectal adenocarcinoma	Wildtype
SW480	Colorectal adenocarcinoma	Wildtype
HT29	Colorectal adenocarcinoma	Wildtype
Onco-DG-1	Thyroid carcinoma	Wildtype
CAL-62	Thyroid anaplastic carcinoma	Wildtype
BHT-101	Thyroid anaplastic carcinoma	Wildtype
8305C	Thyroid carcinoma	Wildtype

Amplification and sequencing of *p21B*

Amplification of the coding region of *p21B* was performed using forward primer *GCTGCATGGACTCTGTGATC* and reverse primer *AGGCAACTGAAGGGCAGG*. The same primers were used to amplify both cDNA and genomic DNA. PCR amplification was carried out using Dynazyme EXT DNA polymerase (FINN-ZYMES, Espoo, Finland) in a 50 µl reaction mix containing 1× PCR buffer, 1.5 mM MgCl₂, 0.5 mM of each deoxynucleotide triphosphate, 5% DMSO, 0.2 µM of each primer and DNA template (0.5 µl cDNA or 1 µl genomic DNA). The PCR conditions were an initial denaturation step of 5 min at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C, and a final elongation step of 7 min at 72°C. DNA sequencing was performed using the same primers as for PCR amplification. Capillary electrophoresis, data collection and sequence analysis were performed on an automated DNA sequencer (ABI 3700).

Haplotype analysis

Haplotype analysis was performed using the Haploview software.²³

Statistical analysis

All statistical calculations were performed using the Primer of Biostatistics system, version 5.0.

Results

We previously sequenced the *p21^{WAF1/CIP1}* and *p21B* coding regions in 73 tumor samples from patients with locally advanced breast cancer and observed no somatic mutations.¹⁷ We here extend the study by sequencing the complete coding region of *p21^{WAF1/CIP1}* and *p21B* in tumor samples from a total of 320 patients with locally advanced breast cancer (including the 73 from the first study) and 201 patients with metastatic breast cancer. Among the 521 tumor samples analyzed, no mutation affecting the coding region of *p21^{WAF1/CIP1}* was observed. Considering *p21B*, one point mutation was discovered. This was a G to A transition of nucleotide number 325 in the *p21B* reading frame, leading to an aspartate to asparagine change in codon 109 (nucleotide numbering according to GenBank accession Z85996 and ORF described by Nozell and Chen 2002.¹) The mutation was found in a patient suffering from metastatic breast cancer. These results indicate that mutations affecting *p21B* are rare in breast cancer.

No mutation of *p21B* was detected in a panel of 20 immortalized cell lines of human origin (Table I).

TABLE II – PREVALENCE OF *p21B^{G128T}*

	Number of observations	Number of individuals analyzed	Heterozygote frequency
Stage I/II breast cancer	7	132	0.053
Locally advanced breast cancer	20	358	0.056
Metastatic breast cancer	15	201	0.075
Total breast cancer	42	691	0.061
Healthy controls	49	1,015	0.048

Several polymorphisms have been detected in the *p21^{WAF1/CIP1}* transcriptional region. Recently, we reported that one of these variants, *p21^{G251A}*, was not associated with breast cancer risk in general, but with development of locally advanced breast cancer in particular.¹⁸ During the screening of the complete coding region of *p21B*, we observed a novel polymorphism in our tumor samples, in addition to the previously reported *p21B^{T35C}*.¹⁷ This novel polymorphism, a G to T transversion of nucleotide 128, leading to a glycine to valine change of codon 43 (designated *p21B^{G128T}*), was confirmed not to be a somatic mutation by the detection of its presence also in leukocytes from the cancer patients.

Haplotype analysis revealed *p21B^{G128T}* to be randomly distributed among individuals with respect to distribution of other polymorphisms in the *CDKN1A* locus; thus, it was not forming a distinct haplotype with *p21B^{T35C}* or with any of the polymorphisms previously observed in the *p21^{WAF1/CIP1}* coding region.¹⁸

To search for potential associations between the *p21B^{G128T}* polymorphism and breast cancer risk, we extended our material and examined the distribution of this polymorphism in blood samples from further 38 patients with locally advanced and 132 with stage I/II breast cancer in addition to 1,015 controls. The results are summarized in Table II. As previously reported for *p21B^{T35C}*,¹⁷ the novel *p21B^{G128T}* was not differently distributed between breast cancer cases and healthy individuals. *p21B^{G128T}* was observed to be heterozygous in 6.1% of the samples from breast cancer patients and in 4.8% of the healthy controls, revealing no significant difference in distribution between the two groups ($p = 0.273$, Fischer exact test). Dividing the samples from breast cancer patients into subclasses of disease, heterozygosity for *p21B^{G128T}* was observed in 5.9% of patients with locally advanced breast cancer and in 7.5% of the patients suffering from metastatic breast cancer, thus, no difference in distribution was observed when comparing locally advanced breast cancer or metastatic breast cancer to the other cancer phenotypes and healthy controls ($p > 0.3$ for all comparisons).

We previously reported mutations of *TP53* to correlate to resistance to chemotherapy *in vivo*.^{19,20} Since *p21^{WAF1/CIP1}* and *p21B* are downstream genes transcriptionally regulated by p53, and linked to the key cellular events growth arrest and apoptosis, respectively, polymorphisms affecting the function of these transcripts may potentially influence the function of the p53 pathway. Previously, we showed that there was no correlation between therapy outcome and occurrence of either *p21B^{T35C}* or any of the polymorphisms affecting *p21^{WAF1/CIP1}*.¹⁷ Here, we found the novel *p21B^{G128T}* not to be associated with therapy failure to anthracycline ($n = 183$ patients) or mitomycin-containing chemotherapy ($n = 37$ patients), and none of the polymorphisms were associated with therapy failure to paclitaxel monotherapy ($n = 100$ patients) for locally advanced breast cancer.

Discussion

While *p21^{WAF1/CIP1}* plays a key role in cell cycle control acting as a main downstream effector of p53-induced growth arrest, the frequency of genetic alterations in this gene has been found to be low in different human cancers.^{2–7,24} Considering polymorphisms, in general they have not been found associated with cancer risk or

tumor behavior, except for $p21^{C93A}$ which remains controversial.^{6,8-16,25} Interestingly, in a recent study we found the $p21^{G251A}$ polymorphism, leading to an arginine to glutamine change of codon 84, to be associated with risk of developing locally advanced breast cancer but not to overall breast cancer risk.¹⁸ An explanation could be that this change is not associated with tumor risk per se but eventually may cause an enhanced growth rate of established cancers. This illustrates the need for different endpoints when evaluating different tumor characteristics for such studies.

Recently, an alternative transcript from the *CDKN1A* locus, $p21B$, was identified. Similar to $p21^{WAF1/CIP1}$, $p21B$ is transcriptionally induced by p53. However, in contrast to $p21^{WAF1/CIP1}$, $p21B$ was found to induce apoptosis.¹ So far no studies apart from our previous report¹⁷ have examined genetic disturbances in $p21B$ in human cancer tissue.

The findings here of only one single mutation affecting $p21B$ and no mutations affecting $p21^{WAF1/CIP1}$ in a total of 521 breast tumors sequenced for the complete coding region of both transcripts suggests mutations affecting these two gene products to be a rare event in breast cancer.

We have identified two polymorphisms affecting $p21B$, $p21B^{T35C,17}$ and $p21B^{G128T}$. Distribution of these polymorphisms did not create a particular haplotype, and none of them was found

associated with any of the previously identified $p21^{WAF1/CIP1}$ polymorphisms. Further, both polymorphisms were found to be equally distributed among breast cancer and non-breast cancer individuals. In contrast to the recently identified $p21$ polymorphism, $p21^{G251A}$, we found the two polymorphisms affecting $p21B$ also to be equally distributed among locally advanced, primary operable and metastatic breast cancer, and none of the polymorphisms were associated with chemotherapy outcome.

We conclude that mutations of $p21B$ as well as $p21^{WAF1/CIP1}$ are rare events in breast cancer. Together with the observation that none of the two identified $p21B$ polymorphisms correlated to breast cancer risk or breast cancer therapy outcome, our findings do not suggest a major role of the $p21B$ transcript to breast carcinogenesis or clinical behavior of established breast cancers in humans.

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References

- Nozell S, Chen X. $p21B$, a variant of $p21(Waf1/Cip1)$, is induced by the p53 family. *Oncogene* 2002;21:1285-94.
- Ibrahim SO, Lillehaug JR, Dolphine O, Johnson NW, Warnakulasuriya K, Vasstrand EN. Mutations of the cell cycle arrest gene $p21(WAF1)$, but not the metastasis-inducing gene $S100A4$, are frequent in oral squamous cell carcinomas from Sudanese toombak dippers and non-snuff-dippers from the Sudan, Scandinavia, USA and UK. *Anticancer Res* 2002;22:1445-51.
- Park YE, Choi KC, Choi YH. $p21$ expression and mutation in gastric carcinoma: analysis by immunohistochemistry and PCR-SSCP. *J Korean Med Sci* 1998;13:507-12.
- Qari MH, Khalil SH, Kambouris M, Meyer BF. Mutation of p16, $p21$ or cyclin dependent kinase 4 is rare in acute lymphoblastic leukaemia. *Br J Haematol* 1998;103:467-72.
- Shiohara M, Koike K, Komiya A, Koeffler HP. $P21(WAF1)$ mutations and human malignancies. *Leuk Lymphoma* 1997;26:35-41.
- Sun Y, Hildesheim A, Li H, Li Y, Chen JY, Cheng YJ, Hayes RB, Rothman N, Bi WF, Cao Y, Yao KT, Lanier AP, Hegamyer G, El-Deiry WS, Xiong Y, Colburn NH. No point mutation but a codon 31ser→arg polymorphism of the $WAF1/CIP1/p21$ tumor suppressor gene in nasopharyngeal carcinoma (NPC): the polymorphism distinguishes Caucasians from Chinese. *Cancer Epidemiol Biomarkers Prev* 1995;4:261-7.
- Tsumanuma I, Tanaka R, Abe S, Kawasaki T, Washiyama K, Kumazaki T. Infrequent mutation of $Waf1/p21$ gene, a CDK inhibitor gene, in brain tumors. *Neurol Med Chir (Tokyo)* 1997;37:150-6 (discussion 6-7).
- Chen 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.
- Facher EA, Becich MJ, Deka A, Law JC. Association between human cancer and two polymorphisms occurring together in the $p21Waf1/Cip1$ cyclin-dependent kinase inhibitor gene. *Cancer* 1997;79:2424-9.
- Harima Y, Sawada S, Nagata K, Sougawa M, Ostapenko V, Ohnishi T. Polymorphism of the $WAF1$ gene is related to susceptibility to cervical cancer in Japanese women. *Int J Mol Med* 2001;7:261-4.
- Hsieh YY, Tsai FJ, Chang CC, Chen WC, Tsai CH, Tsai HD, Lin CC. $p21$ gene codon 31 arginine/serine polymorphism: non-association with endometriosis. *J Clin Lab Anal* 2001;15:184-7.
- Ressiniotis T, Griffiths PG, Keers SM, Chinnery PF, Birch M. A polymorphism at codon 31 of gene $p21$ is not associated with primary open angle glaucoma in Caucasians. *BMC Ophthalmol* 2005;5:5.
- Roh J, Kim M, Kim J, Park N, Song Y, Kang S, Lee H. Polymorphisms in codon 31 of $p21$ and cervical cancer susceptibility in Korean women. *Cancer Lett* 2001;165:59-62.
- Sjalander A, Birgander R, Rannug A, Alexandrie AK, Tornling G, Beckman G. Association between the $p21$ codon 31 A1 (arg) allele and lung cancer. *Hum Hered* 1996;46:221-5.
- Su L, Liu G, Zhou W, Xu LL, Miller DP, Park S, Lynch TJ, Wain JC, Christiani DC. No association between the $p21$ codon 31 serine-arginine polymorphism and lung cancer risk. *Cancer Epidemiol Biomarkers Prev* 2003;12:174-5.
- Tsai MH, Chen WC, Tsai FJ. Correlation of $p21$ gene codon 31 polymorphism and TNF- α gene polymorphism with nasopharyngeal carcinoma. *J Clin Lab Anal* 2002;16:146-50.
- Staalesen V, Leirvaag B, Lillehaug 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.
- Staalesen V, Knappskog S, Chrisanthar R, Nordgard SH, Lokkevick E, Anker G, Ostenstad B, Lundgren S, Risberg T, Mjaaland I, Gram IT, Kristensen VN, Borresen-Dale AL, Lillehaug JR, Lonning PE. The novel $p21$ polymorphism $p21G251A$ is associated with locally advanced breast cancer. *Clin Cancer Res* 2006;12:6000-4.
- Geisler S, Borresen-Dale AL, Johnsen H, Aas T, Geisler J, Akslen LA, Anker G, Lonning PE. TP53 gene mutations predict the response to neoadjuvant treatment with 5-fluorouracil and mitomycin in locally advanced breast cancer. *Clin Cancer Res* 2003;9:5582-8.
- Geisler S, Lonning PE, Aas T, Johnsen H, Fluge O, Haugen DF, Lillehaug JR, Akslen LA, Borresen-Dale 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.
- Helle SI, Ekse D, Holly JMP, Lonning PE. The IGF-system in healthy pre- and postmenopausal women: relations to demographic variables and sex-steroids. *J Steroid Biochem Mol Biol* 2002;81:95-102.
- Gram IT, Bremnes Y, Ursin G, Maskarinec G, Bjurstam N, Lund E. Percentage density, Wolfe's and Tabar's mammographic patterns: agreement and association with risk factors for breast cancer. *Breast Cancer Res* 2005;7:R854-R861.
- Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263-5.
- Terry LA, Boyd J, Alcorta D, Lyon T, Solomon G, Hannon G, Berchuck A, Beach D, Barrett JC. Mutational analysis of the $p21/WAF1/CIP1/SDI1$ coding region in human tumor cell lines. *Mol Carcinog* 1996;16:221-8.
- Shih CM, Lin PT, Wang HC, Huang WC, Wang YC. Lack of evidence of association of $p21WAF1/CIP1$ polymorphism with lung cancer susceptibility and prognosis in Taiwan. *Jpn J Cancer Res* 2000;91:9-15.