A Novel Type of p53 Pathway Dysfunction in Chronic Lymphocytic Leukemia Resulting from Two Interacting Single Nucleotide Polymorphisms within the p21 Gene

Gillian G. Johnson,1,3 Paul D. Sherrington,1,3 Anthony Carter,1 Ke Lin,1,3 Triantafillos Liloglou,2 John K. Field,2 and Andrew R. Pettitt1,3

Divisions of 1Haematology and 2Surgery and Oncology, University of Liverpool School of Cancer Studies; 3Department of Haematology, Royal Liverpool University Hospital, Liverpool, United Kingdom

Abstract

The ATM-p53 pathway plays an important role in the biology of chronic lymphocytic leukemia (CLL). Its functional integrity can be probed by exposing CLL cells to ionizing radiation (IR) and measuring levels of p53 protein and one of its transcriptional targets, the cyclin-dependent kinase inhibitor p21. We have previously identified two abnormal p53/p21 response patterns associated with inactivating mutations of TP53 and ATM, respectively. Here, we describe a third abnormal response pattern characterized by failure of p21 protein accumulation despite a normal p53 protein response. This so-called "type C" response was detected in 10.6% of unselected patients and was associated with resistance of CLL cells to p53-dependent killing by IR, with the clinically more aggressive variant of CLL characterized by unmutated immunoglobulin heavy-chain genes and with a single nucleotide polymorphism at codon 31 of the p21 gene in which Ser is replaced by Arg. Arg. CLL samples with this allelic variant displayed impaired IR-induced up-regulation of total p21 mRNA and did not express the Arg-encoding transcript, except in those cases harboring an additional single nucleotide polymorphism (T instead of C) in the 3′-untranslated region of the same p21 allele. Our data provide new insight into the importance of p21 in CLL biology. [Cancer Res 2009;69(12):5210–17]

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of clonal mature B cells expressing CD19, CD5, and CD23 and reduced levels of membrane IgM, IgD, and CD79b (1). The disease runs a chronic relapsing course that varies considerably between patients. Research into understanding this clinical variability has led to the identification of several prognostic biomarkers that can identify subgroups of patients who are at high risk of rapid disease progression, poor response to therapy, and shortened survival. These biomarkers include mutational status of the immunoglobulin heavy-chain gene (IgVH; refs. 2, 3), surface expression of CD38 (2, 3), intracelluar expression of ZAP-70 (4–6), and chromosomal aberrations (7). The most ominous predictor of adverse outcome is deletion/mutation of the TP53 gene on chromosome 17p13 (8–16).

p53, the protein encoded by the TP53 gene, is a transcription factor that is present at low levels under resting conditions owing to its short half-life but which becomes activated following DNA damage. Activation, which occurs predominantly by phosphorylation, prolongs the half-life of p53 protein and allows it to accumulate in the nucleus where it regulates the expression of a wide range of genes that induce apoptosis, cell cycle arrest, and DNA repair (17). In this way, p53 plays a pivotal role in limiting clonal expansion, maintaining genomic stability, and mediating the action of DNA-damaging chemotherapy (18–21). The TP53 gene is inactivated by mutation/deletion in >50% of human cancers and this has consistently been linked to short survival and poor response to chemotherapy (22, 23).

One of the most important transcriptional targets of p53 is p21, a cyclin-dependent kinase inhibitor belonging to the Cip/Kip family (24). p21 was the first identified inhibitor of cyclin/cyclin-dependent kinase complexes (25, 26). p21 binds to cyclin-dependent kinase complexes via its NH2-terminal domain and inhibits their kinase activity, thereby arresting cells in the G1, G2-M phases of the cell cycle. p21 also has other important functions including the regulation of apoptosis. Thus, the NH2-terminal domain of p21 has been shown to bind to procaspase-3, thereby blocking its processing and activation and protecting cells from Fas-induced apoptosis (27). On the other hand, overexpression of p21 has been shown to enhance the cytotoxic effect of cisplatin in human ovarian carcinoma cells (28).

Using ionizing radiation (IR) to probe the functional integrity of the p53 pathway in cultured CLL cells, we have previously established that p53 dysfunction can result not only from loss of wild-type p53 but also from mutation of ATM, which is required for the activation of p53 following double-strand DNA breaks (29, 30). In this assay, irradiated CLL cells are analyzed for up-regulation of p53 and p21 proteins, the latter serving as a readout of p53 functional activity. Cells with a functionally normal ATM-p53-p21 pathway have low basal levels of p53 and p21, which both increase following treatment with IR. Type A p53 dysfunction, associated with TP53 gene mutations, is characterized by high basal levels of p53 protein and impaired IR-induced up-regulation of p21. In contrast, cases with type B p53 dysfunction have low basal levels of p53 and p21 and impaired up-regulation of both proteins following exposure to IR. This type of p53 dysfunction was found to be associated with mutations in the ATM gene (29). Both types of p53 dysfunction are associated with shorter patient survival and other adverse prognostic indicators, such as unmutated IgVH genes, usage of the V<sub>j</sub>3-21 gene segment, and adverse chromosomal abnormalities (31–33).

Screening of consecutive CLL patients presenting to our center has identified a further p53/p21 response to IR (called the "type C"
Materials and Methods

Type C defect and to identify any biological/clinical associations. The aim of the present study was to investigate the cause of the type C defect and to identify any biological/clinical associations. For pragmatic reasons, we chose to analyze single nucleotide polymorphisms (SNP) in the TP53 and p21 genes as a starting point for our investigations.

SNPs are single base-pair changes that occur in germ-line DNA during the course of evolution and that contribute to the traits that define individuals as unique (34). The presence of SNPs in some genes may predispose to certain diseases and influence therapeutic response and clinical outcome (35, 36). SNPs have been identified in the TP53 and p21 genes at a frequency that depends on race and geography (37). The human TP53 gene contains at least 10 different SNPs in the coding and noncoding regions, and some of these have been shown to influence the function of p53 protein (38-42). In addition, at least 6 SNPs have been identified in the coding and noncoding regions of the human p21 gene (42, 43). These TP53 and p21 SNPs have been associated with a range of human cancers in different populations (42).

In lung cancer in Caucasian populations, SNPs at codon 72 of TP53 (C-to-G resulting in a Pro-to-Arg substitution) and codon 31 of p21 (C-to-A resulting in a Ser-to-Arg substitution) are associated with low basal levels of p53 and p21 and failure of p21 to up-regulate despite a normal p53 protein response. The type C defect (that is characterized by low basal levels of p53 and p21 and p21 noncoding regions of the human TP53 gene (42, 43). These TP53 and p21 SNPs have been associated with certain diseases and influence therapeutic response and clinical outcome (35, 36). SNPs have been identified in the TP53 and p21 genes at a frequency that depends on race and geography (37). The human TP53 gene contains at least 10 different SNPs in the coding and noncoding regions, and some of these have been shown to influence the function of p53 protein (38-42). In addition, at least 6 SNPs have been identified in the coding and noncoding regions of the human p21 gene (42, 43). These TP53 and p21 SNPs have been associated with a range of human cancers in different populations (42).

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CLL patients. The project was approved by the Liverpool Research Ethics Committee and clinical samples were obtained with written informed consent. All patients had typical CLL (CD19+, CD5-, CD23+, and weak light-chain-restricted surface immunoglobulin). Mononuclear cells were prepared from whole blood by centrifugation on Lymphoprep (Nycomed) and cryopreserved in 10% DMSO (Sigma UK).

CLL cell culture. Cells were thawed and washed in PBS, and cultured (37°C with 5% CO2) in RPMI (Invitrogen) with 1% bovine serum albumin (Sigma) in plates coated with poly(2-hydroxyethyl methacrylate) (Sigma) to prevent adhesion.

Measurement of p53/p21 protein levels by flow cytometry. p53 and p21 protein levels were measured in irradiated and nonirradiated CLL cells as described previously by Carter and colleagues (30).

DNA isolation. Cells were thawed and washed in PBS. DNA was isolated using the Promega Wizard Genomic DNA purification kit (Promega). Detection of SNPs by PCR-RFLP analysis. PCR-RFLP analysis was used to identify the TP53 codon 72 and p21 codon 31, codon 149, and 3'-UTR SNPs. PCR amplification was carried out from genomic DNA with Taq polymerase and the supplied buffer (Promega) plus 3 mmol/L MgCl2, 0.2 mmol/L deoxynucleotide triphosphates (Promega), and 0.4 pmol/μL of each forward and reverse primer (Supplementary Table S1). The PCR conditions used were 1 cycle at 94°C for 5 min; 30 cycles of 94°C for 45 s, 59°C or 60°C for 45 s, and 72°C for 45 s; and 1 cycle at 72°C for 10 min. Following amplification, PCR products were purified and digested using the restriction enzymes detailed in Supplementary Table S1. The digested products were separated on a 3% agarose gel and visualized with a Fuji Film FLA-5000 imager (Fuji Film).

Pyrosequencing. PCR amplification from genomic DNA of the p21 promoter SNP was carried out using HotStart Taq polymerase and supplied buffer (Qiagen), 0.2 mmol/L deoxynucleotide triphosphates, and 0.2 mmol/L of primers 5'-GGGCCTGATTTGGCCCTT-3' and 5'-GGAGCC-CAAGGACAA-3' using 95°C for 4.5 min, 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles and then 72°C for 10 min. The quality and quantity of the PCR products was assessed by agarose gel electrophoresis before cleanup and pyrosequencing analysis. For the latter, the PCR product was incubated with 10 pmol/μL sequencing primer (5'-GTGGGGATGACGATTCCT-3') and then processed and analyzed on a PSQ96MA machine (Biotage).

Quantitative Real-time PCR for p21, MDM2, PIG3, and L27. Total RNA was extracted using a Trizol/chloroform method. cDNA was synthesized from RNA using ImPromII Reverse Transcription System (Promega) and an oligo(dT)16 primer. All real-time PCRs were carried out in duplicate using an Opticon2 thermal cycler (MJ Research) alongside no-template controls. The primers used are detailed in Supplementary Table S2. Each 25 μL reaction included 12.5 μL DyNamo master mix (Finzymes) and 10 pmol of each relevant primer. Reactions were incubated at 95°C for 10 min, 94°C for 30 s, 61°C for 30 s, and 72°C for 30 s for 45 cycles. Following each extension step, the fluorescence was measured at 76°C, 78°C, and 80°C. The reactions were incubated at 72°C for 10 min, and to determine melting temperatures, the fluorescence was measured at 1 s intervals while the temperature was increased from 65°C to 98°C in 0.5°C steps. The relative levels of p21, MDM2, and PIG3 transcripts compared with L27 were calculated.

IR treatment. Cells were irradiated with 9 Gy IR using a cesium 137 source as described previously (29, 30).

Measurement of cell viability with DiOC6/propidium iodide. Cultured cells were added to DiOC6 (40 mmol/L; Calbiochem). Following incubation at 37°C for 20 min, an equal volume of propidium iodide (10 mg/mL; Sigma) was added and the cells were incubated on ice for a further 30 min. Analysis by flow cytometry was carried out. Live cells are detected as DiOC6 bright/propidium iodide dim, whereas dead cells are DiOC6 dim/propidium iodide bright.

Transformations. For transcript bias experiments, p21 was amplified from cDNA using primers 5’-GTCAAGACGGTCTGGTTG-3’ and 5’-CTCTGCCAATCCATACGCGG-3’. The PCR product was ligated into a pGEM-T vector (Promega) and transformed into JM109 competent cells. Plasmid DNA (from 10 colonies per sample) was digested with EcoRI and BflI (New England Biolabs). Digestions were separated on a 3% agarose gel and visualized.

For genotyping of the p21 alleles, PCRs were done on genomic DNA using Taq polymerase and the supplied buffer (Promega) plus 3 mmol/L MgCl2, 0.2 mmol/L deoxynucleotide triphosphates (Promega), and 0.4 pmol/μL primers (5'-GTCAAGACGGTCTGGTTG-3’ and 5’-CCAGGTATGTGACATCGGAG-3’) using 94°C for 5 min, 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s for 30 cycles and 72°C for 10 min. Purified PCR products were ligated into a pGEM-T vector and transformed into JM109 competent cells. PCR-RFLP analysis was carried out on plasmid DNA to determine the codon 31 and 3’-UTR genotype of each colony.

Clinical data. Clinical data were obtained and stored in accordance with data protection legislation. Survival probabilities were estimated by Kaplan-Meier survival curves and comparisons were made using the log-rank test. Biological variables were compared using the appropriate statistical test.

Results

Definition of the type C p53 pathway defect. Since 2004, new research samples obtained from CLL patients attending the Royal Liverpool University Hospital have been screened for functional defects of the ATM-p53-p21 pathway using our previously reported flow cytometric method (30). We observed a hitherto unrecognized response to IR characterized by impaired up-regulation of p21 in the context of a normal p53 protein response (Fig. 1). We have called this response the "type C" defect.

The type C defect is associated with Ser/Arg at the p21 codon 31 SNP site. Because SNPs in TP53 codon 72 and p21 codon...
31 have previously been associated with low p21 mRNA levels in lung cancer (44), we speculated that they might also account for the type C response to IR in CLL. To address this question, both SNPs were analyzed in 31 selected CLL samples, 16 with a normal p53/p21 response to IR, and 15 with the type C defect.

Analysis of the TP53 codon 72 SNP showed no association between the SNP genotype and the p53/p21 functional response (Table 1). In contrast, analysis of the p21 codon 31 SNP revealed a significant association between the Arg variant and the type C phenotype (Table 1). Thus, 8 of the 15 cases with the type C response had Ser/Arg at p21 codon 31 compared with only 2 of the 16 cases with a normal p53/p21 response ($P = 0.023$).

Three other known p21 SNPs of potential functional significance were also analyzed in this cohort of patients. The SNP in the 3′-UTR of p21 is 20 nucleotides from the stop codon (45). 3′-UTRs have been shown to be important for RNA stability and gene regulation (46, 47). Therefore, this SNP could potentially explain the type C response in CLL. Of the 30 available cases that

![Figure 1. Definition of specific p53/p21 responses to IR. Flow cytometry histograms showing a normal p53/p21 response to IR, the type A defect associated with TP53 mutation, the type B defect associated with ATM mutation, and the previously unreported type C defect. Darker and lighter lines, irradiated and unirradiated cells, respectively. Staining intensity for p53 and p21 is measured using mean fluorescence intensity values. p53 dysfunction is defined as failure of p21 to increase by at least 25% in irradiated cells. The type A defect is characterized by baseline p53 expression of at least 15 mean fluorescence intensity units. The type B defect is characterized by baseline p53 expression of <15 mean fluorescence intensity units and failure of p53 to increase by at least 80% in irradiated cells. The type C defect is characterized by baseline p53 expression of <15 mean fluorescence intensity units and an increase in p53 of at least 80% in irradiated cells.](image-url)
were screened, only 3 were C/T and none were T/T. There was no association between the SNP genotype and the p53/p21 response to IR (Table 1). Interestingly, however, each of the 3 cases with C/T association between the SNP genotype and the p53/p21 response were screened, only 3 were C/T and none were T/T. There was no association between the p21 with skin cancer (43). Using pyrosequencing, the variant has been shown to be significantly increased in patients able on this SNP and its role in cancer, but the frequency of the G 5 bp upstream of a p53 binding site (43). Little information is available on the allelotypic profile of p21 3′-UTR SNP and the p53/p21 response to IR (Table 1). This was in keeping with its reported rarity in Caucasian populations (42).

The SNP in the promoter region of p21 is an A-to-G transition 5 bp upstream of a p53 binding site (43). Little information is available on this SNP and its role in cancer, but the frequency of the G variant has been shown to be significantly increased in patients with skin cancer (43). Using pyrosequencing, the p21 promoter SNP was examined in all 31 CLL samples; however, there was no association between the p21 promoter SNP and the p53/p21 response to IR (Table 1).

CLL samples with Ser/Arg at p21 codon 31 display a Ser transcript bias. We next sought to establish why the type C Ser/Arg cases displayed lower levels of p21 mRNA compared with Ser/Ser cases. One possible explanation is that the p21 transcript derived from the Arg-encoding allele is present at lower levels than that derived from the Ser-encoding allele. To address this question, the region of the p21 transcript containing codon 31 was cloned from the Ser/Arg cases and the clones were analyzed for their codon 31 SNP status. For each case, 20 colonies were selected, 10 derived from the Ser-encoding allele and 10 from nonirradiated cells. Because the findings were similar irrespective of whether or not the cells had been irradiated, the results from nonirradiated and irradiated cells were pooled.

All 9 Ser/Arg cases for which stored material was available were analyzed. In all 7 cases with the type C defect, only the p21 codon 31 Ser transcript was detected (Table 2). In contrast, Ser and Arg transcripts were detected at comparable levels in the 2 cases with a normal p53/p21 response to IR (cases 10 and 21; Table 2). Interestingly, both of the latter samples were C/T at the p21 3′-UTR SNP site, whereas the 7 Ser/Arg cases with the type C defect and a transcript bias were all C/C. These findings suggest that the impaired accumulation of total p21 mRNA in type C cases with Ser/Arg at p21 codon 31 results from instability of the Arg-encoding p21 transcript, which is overcome by coinheritance of C/T at the 3′-UTR SNP site. Blocking translation with cycloheximide had no effect on the allelotypic profile of p21 codon 31 transcripts (data not shown), indicating that absence of the Arg transcript was not due to translation-coupled degradation.

### Table 1. Association of the type C defect and selected SNPs in TP53 and p21

<table>
<thead>
<tr>
<th>SNP</th>
<th>Nucleotide variant</th>
<th>Amino acid variant</th>
<th>Normal p53/p21 response</th>
<th>Type C p53/p21 response</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21 codon 31</td>
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<td>Ser/Ser</td>
<td>14</td>
<td>7</td>
<td>21</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>C/A</td>
<td>Ser/Arg</td>
<td>2</td>
<td>8</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>Arg/Arg</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>p21 codon 149</td>
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<td>12</td>
<td>24</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>A/G</td>
<td>Asp/Gly</td>
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<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>Gly/Gly</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>p21 3′-UTR</td>
<td>C/C</td>
<td>NA*</td>
<td>14</td>
<td>13</td>
<td>27</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
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<td>0</td>
<td>0</td>
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<tr>
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<td>10</td>
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<tr>
<td></td>
<td>G/A</td>
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<td>5</td>
<td>9</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>A/A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td>TP53 codon 72</td>
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<td>Arg/Arg</td>
<td>9</td>
<td>8</td>
<td>17</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td></td>
<td>G/C</td>
<td>Arg/Pro</td>
<td>7</td>
<td>6</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>Pro/Pro</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*NA, nonapplicable.
Frequency of p21 codon 31 SNPs and validation of results in a separate cohort of unselected patients. To ascertain the true frequency of the p21 codon 31 SNP variants in CLL and validate the observations made in the initial cohort, the SNP genotypes were determined in a separate cohort of 105 randomly selected CLL cases. The results are summarized in Table 3. Thirteen of 105 (12.4%) patients were Ser/Arg at codon 31, the remainder being Ser/Ser. The p53/p21 functional status was available for 94 of the 105 cases, of which 10 (10.6%) had the type C defect. There was a highly significant association between the type C defect and Ser/Arg at p21 codon 31 (P = 0.0003).

The p21 codon 31 SNP genotype was also determined by pyrosequencing in 493 samples from healthy individuals from the same predominantly Caucasian population (Table 3). There was no difference in the frequency of the Ser/Arg genotype in the control population versus the CLL cohort (P = 0.9172). Taken together, these findings confirm that the p21 codon 31 Arg variant is associated with the type C phenotype but not with CLL per se.

T at the p21 3′-UTR SNP negates the effect of the p21 codon 31 Arg variant in lowering p21 mRNA levels. The results presented thus far support the idea that the Arg variant at p21 codon 31 produces the type C defect through lowering of p21 mRNA levels and that this effect is overcome by coinheritance of C/T at the 3′-UTR. Seven of the 105 randomly selected CLL samples in the second cohort were Ser/Arg at p21 codon 31 but did not exhibit the type C response (Table 3). We therefore predicted that these cases might also have C/T at the 3′-UTR. To test this hypothesis, all 13 Ser/Arg cases in the second cohort were analyzed for their p21 3′-UTR SNP status by PCR-RFLP. In keeping with our prediction, all 6 Ser/Arg cases with the type C defect were C/C at the 3′-UTR, whereas all 7 non-type C Ser/Arg cases were C/T (Supplementary Table S3). We next examined 3 of these latter cases for a Ser transcript bias. Up to 20 individual clones were analyzed using PCR-RFLP analysis. As expected, all 3 cases examined expressed both Ser-encoding and Arg-encoding p21 transcripts (Supplementary Table S3).

Figure 2. Biological associations of the type C defect and p21 codon 31 allelotype. A, fold increase in p21 mRNA 18 h after treatment of CLL cells with IR. B, fold increase of MDM2 mRNA 18 h after treatment of CLL cells with IR. C, fold increase in PIG3 mRNA 18 h after treatment of CLL cells with IR; only 20 of the 31 original samples were available for this analysis. D, cell death induced by IR (5 Gy) after 48 h.
was no significant difference in IR-induced killing between type C apoptosis in CLL cells and indicate that the type C defect is associated with resistance to such killing irrespective of the underlying cause of the defect.

The type C defect is associated with unmutated IgVH genes irrespective of p21 SNP status. To investigate possible clinical associations of the type C defect, p53 functional status was related to a range of prognostically important biological variables (Table 4). One hundred three cases with available information were included in the analysis, comprising 78 cases with a normal p53/p21 response to IR and all 25 cases with the type C defect (15 cases from the selected cohort plus 10 from the unselected cohort). The combined cohort had typical characteristics, and the median time between diagnosis and sampling for analysis was 6 months. There was no significant difference between the two groups in terms of age, sex, clinical stage, WBC count, CD38 positivity, or adverse chromosomal abnormalities. There was, however, a significant difference in the extent of IgVH mutation. Thus, cases with a normal p53/p21 response had a median of 4.8% IgVH mutation compared with 1.1% in the type C cases ($P = 0.04$). Using a cutoff of 2% mutation, the type C patients were almost twice as likely to have unmutated IgVH genes ($P = 0.04$). In keeping with this observation, analysis of disease kinetics showed a trend toward earlier treatment in the type C group (Supplementary Fig. S1). The association between the type C defect and unmutated (<2%) IgVH genes was independent of p21 SNP status and strengthened when patients with 17p- or 11q- were excluded from analysis ($P = 0.014$; data not shown).

**Discussion**

In the present study, we report a novel type of ATM-p53-p21 pathway defect that is present in ∼10% of CLL patients and characterized by failure of p21 protein to accumulate following DNA damage despite a normal p53 protein response. We show that this defect is associated with resistance to IR-induced cytotoxicity, unmutated IgVH genes, and the Arg variant of the p21 SNP at codon 31. We further show that cases with the Ser/Arg genotype display impaired up-regulation of total p21 mRNA following DNA damage and a marked transcript bias in favor of the Ser-encoding allele. Finally, we show that these effects are opposed by coinheritance of the less common T variant of the SNP in the 3′-UTR of the same p21 allele.

The absence of the Arg-encoding p21 transcript in cases with the type C defect is both striking and intriguing. The type C defect is associated with unmutated IgVH genes on the same p21 allele. Further experiments were done to establish whether the Arg (codon 31) and T (3′-UTR) variants were co-inherited on the same allele. DNA fragments encompassing the codon 31 and 3′-UTR SNP sites from both p21 alleles were PCR amplified from genomic DNA. The PCR products were cloned and individual clones (up to 4 per case) were genotyped for the two SNPs by PCR-RFLP analysis. In all 7 cases, Arg (codon 31) and T (3′-UTR) were present on the same allele (Supplementary Table S3). This suggests that intraallelic interaction between the two SNP sites regulates the stability of the p21 transcript and consequently the expression of p21 protein.

**Type C defect is associated with resistance to IR-induced killing irrespective of p21 SNP status.** Having examined potential causes of the type C p53 pathway defect in CLL, we next examined possible biological associations. Although p21 is best known for its role in mediating cell cycle arrest, it is notoriously difficult to implicate in the regulation of apoptosis in other cell types (27, 28), we postulated that failure of IR-induced p21 up-regulation in CLL cells with the type C defect might be associated with altered sensitivity to IR-induced killing. To address this question, IR-induced killing was compared in 6 CLL samples with a p53/p21 defect (Table 3). In keeping with our hypothesis, IR-induced killing was significantly impaired in cases with the type C p53/p21 defect ($P = 0.039$, Mann-Whitney $U$ test). However, there was no significant difference in IR-induced killing between type C cases with Ser/Ser at p21 codon 31 versus those with Ser/Arg ($P = 0.754$). Further characterization of the 12 type C cases for TP53 mutation/deletion and IR-induced phosphorylation of ATM at Ser1981 established that their resistance to IR-induced killing could not be attributed to structural defects of the TP53 gene or functional defects of ATM protein (data not shown). These findings strongly implicate a role for p21 in the induction of p53-mediated apoptosis in CLL cells and indicate that the type C defect is associated with resistance to such killing irrespective of the underlying cause of the defect.

**Table 2. Ratio of p21 transcripts containing Ser versus Arg at codon 31 in CLL samples with both alleles**

<table>
<thead>
<tr>
<th>Case</th>
<th>p53 functional status</th>
<th>p21 codon 31</th>
<th>No. transcripts*</th>
<th>p21 3′-UTR</th>
<th>p21 3′-UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ser  Arg</td>
<td></td>
<td>Ser  Arg</td>
</tr>
<tr>
<td>17</td>
<td>Type C</td>
<td>Ser/Arg</td>
<td>19  0</td>
<td>C/C</td>
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<tr>
<td>18</td>
<td>Type C</td>
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<td>C/C</td>
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<td>Type C</td>
<td>Ser/Arg</td>
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<td>C/C</td>
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<tr>
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<td>Type C</td>
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<td>C/C</td>
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</tr>
<tr>
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<td>Type C</td>
<td>Ser/Arg</td>
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<td>C/C</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Type C</td>
<td>Ser/Arg</td>
<td>15  0</td>
<td>C/C</td>
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<tr>
<td>24</td>
<td>Type C</td>
<td>Ser/Arg</td>
<td>10  0</td>
<td>C/C</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Normal</td>
<td>Ser/Arg</td>
<td>8  10</td>
<td>C/T</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Normal</td>
<td>Ser/Arg</td>
<td>9  6</td>
<td>C/T</td>
<td></td>
</tr>
</tbody>
</table>

*Not all of the 20 colonies selected from each case yielded DNA bands on the agarose gel.

**Table 3. Frequency of p21 codon 31 variants in 105 unselected CLL patients and 493 healthy individuals**

<table>
<thead>
<tr>
<th></th>
<th>Ser/Ser</th>
<th>Ser/Arg</th>
<th>Arg/Arg</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>All CLL cases</td>
<td>92</td>
<td>13</td>
<td>0</td>
<td>105</td>
</tr>
<tr>
<td>Normal p53/p21 response</td>
<td>49</td>
<td>5</td>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>Type A dysfunction</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Type B dysfunction</td>
<td>22</td>
<td>2</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Type C dysfunction</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Unknown p53/p21</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Functional status</td>
<td>Normal controls</td>
<td>432</td>
<td>59</td>
<td>2</td>
</tr>
</tbody>
</table>
transcription, and targeting for degradation are regulated by AU-rich elements in the 3′-UTR interacting with RNA-binding proteins (46, 49). The stabilizing influence of the T variant of the 3′-UTR SNP suggests that the two SNPs interact within one another within the p21 transcript to alter its folding and regulate its processing. Functional interaction between these two SNPs has not previously been reported. However, a similar phenomenon has been reported in relation to synonymous SNPs in the human dopamine receptor D2 gene (50), thereby supporting the idea that combinations of SNPs within a single allele can have consequences that differ from those of individual SNPs.

It is noteworthy that the median increase in p21 mRNA was 2.2-fold lower in type C Ser/Arg cases than in type C Ser/Ser cases or those with a normal p53/p21 response to IR. It could be argued that this observation is difficult to explain by lack of expression of the Arg-encoding allele alone, and one could speculate that the Arg-encoding transcript might somehow interact with and destabilize the Ser-encoding transcript. However, it could be misleading to place too much emphasis on the median values given the wide variation observed between individual patients within each group.

The impaired cytotoxic response to IR observed in CLL samples with the type C defect is intriguing because it implies that p21 fulfills a proapoptotic function in CLL cells. This idea is in keeping with previous reports implying a role for p21 in the regulation of apoptosis (51, 52) and has potential therapeutic implications.

With regard to clinical associations, a significant correlation was observed between the type C defect and the clinically more aggressive variant of CLL characterized by unmutated IgVH genes. This observation is particularly intriguing in cases with the p21 codon 31 SNP as it suggests that the SNP, being present in germ-line DNA, might influence the extent of IgVH mutation that occurs in the CLL precursor before clonal expansion. This idea is plausible as there is a potential link between p21 and somatic hypermutation. Thus, p21 is known to regulate proliferating cell nuclear antigen (53–56), which in turn regulates the DNA polymerases responsible for translesion DNA synthesis, the latter being required for somatic hypermutation (56). Alternatively, it is possible that the type C p53 defect plays a facilitatory role in allowing CLL clones with unmutated IgVH genes to expand.

Although the type C defect was strongly associated with Arg at p21 codon 31, only ~50% of type C cases harbored this variant. The remaining type C cases had little or no impairment of p21 mRNA up-regulation following irradiation. This suggested that p21 mRNA was not being translated in these cases. Two proteins, CRT and CUGBP1, are known to bind to p21 mRNA and control p21 translation and protein expression (57). However, analysis of these proteins by Western blotting showed no association with the type C defect in cases with Ser/Ser at p21 codon 31 (data not shown). The cause of this putative translational uncoupling therefore remains unclear.

In summary, our findings indicate that impaired p21 protein accumulation in the context of a normal p53 protein response to DNA damage has important biological consequences for CLL cells and can arise through more than one mechanism, including SNPs in the p21 gene that interact with one another. Further studies using large cohorts of uniformly treated patients are required to investigate the potential importance of the type C defect and p21 SNPs as determinants of clinical outcome.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### References


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Gillian G. Johnson, Paul D. Sherrington, Anthony Carter, et al.


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