DNA Damage-associated Dysregulation of the Cell Cycle and Apoptosis Control in Cells with Germ-line p53 Mutation

Kumiko Goi, Masatoshi Takagi, Satoshi Iwata, Domenico Delia, Minoru Asada, Rosangela Donghi, Yukiko Tsunematsu, Shinpei Nakazawa, Hiroshi Yamamoto, Jun Yokota, Kazuo Tamura, Yoshifumi Saeki, Joji Utsunomiya, Takashi Takahashi, Ryuzu Ueda, Chikashi Ishioka, Mariko Eguchi, Nanao Kamata, and Shuki Mizutani

Department of Virology, The National Children's Medical Research Center, 3-35-35 Taishido, Setagaya-ku, Tokyo 154, Japan (K. G., M. T., S. I., S. M., M. A.); Division Oncologia Sperimentale, Instituto Nazionale Tumori, Via G. Venezian 1, 10133 Milan, Italy (D. D., R. D.); Department of Hematology, The National Children's Hospital, 3-35-35 Taishido, Setagaya-ku, Tokyo 154, Japan (T. Y.); Department of Pediatrics, Yamanashi Medical University, 1110 Shimokawaguchiko, Daito-cho, Nakakama-Gun, Yamanashi 409-38, Japan (S. N.); Department of Surgery, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku 104, Tokyo, Japan (H. Y.); Biology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku 104, Tokyo, Japan (Y. Y.); Laboratory of Chemistry, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan (T. T. R. U.); Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980, Japan (C. I.); and Department of Cancer Cytogenetics, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan (M. E., N. K.)

ABSTRACT

Lymphoblastoid cell lines (LCLs) with heterozygous p53 mutations at residues 286A, 133R, 282W, 132E, and 213ter were established from five independent Li-Fraumeni syndrome families. When cell cycle regulation in response to γ-irradiation was studied, these LCLs showed an abnormal G1 checkpoint associated with defective inhibition of cyclin E/cyclin-dependent kinase 2 activity in all cases except for 282W LCL, which showed a normal G1 checkpoint. On the other hand, the control of S-phase-G2 was determined by cyclin A/cyclin-dependent kinase 2 activity was defective in all these LCLs. The mitotic checkpoint was also defective in the two LCLs analyzed as either competent or incompetent for G1 arrest. When radiation-induced apoptosis, which requires wild-type p53 function under optimal conditions, was studied, all of these LCLs showed significant failure compared to normal LCLs. These findings indicate that although p53-dependent transactivation and G1-S-phase cell cycle control are variably dysregulated, the induction of apoptosis and control of the cell cycle at S-phase-G2 and the mitotic checkpoint in response to DNA-damaging agents are consistently dysregulated in heterozygous mutant LCLs. This suggests that these dysfunctions underlie, at least in part, the susceptibility of Li-Fraumeni syndrome families to cancer. Furthermore, the approach presented is a potentially useful method for studying individual carriers of different germ-line p53 mutations and different biological features.

INTRODUCTION

Human cancer is a multistep process resulting from the mutation of genes involved in the regulation of cell growth, cell differentiation, and apoptosis. The most common target of mutation is the p53 gene, whose product codes for a transcription factor that regulates the expression of genes with an adjacent p53 recognition sequence, such as the MDM2 (1), p21<sup>WAF1</sup> (2), GADD45 (3), muscle-type creatine kinase (4), and Bax (5) genes, and leads to either cell cycle arrest in late G1 (6) or apoptosis in response to DNA damage (7). The loss of p53 function can occur through point mutation, allelic loss, rearrangements, or intragenic deletions in a highly conserved “hot spot” region spanning the middle third of the gene. This usually leads to disruption of specific DNA-binding and transcription, which are apparently critical events in the development or clonal progression of cancer (8).

Germ-line transmission of a mutant p53 gene in families with LFS has revealed a new role for p53 in genetic predisposition to cancer (9, 10). In the presence of a mutated p53, a dominant negative effect against the wild-type p53 gene, (11, 12) or a gain of function effect (13) has been identified in heterozygous cell clones. These diverse effects of p53 mutations seem to be determined by where the mutation resides in the p53 gene and how these mutations affect the relative expression level of wt versus mt p53 protein (14), which may vary depending on the family (15–17). These previous findings suggest that the ability of wt p53 to inhibit the function of wt p53 in LFS patients may be determined both genetically and epigenetically and that studies on the cell biological effect of germ-line p53 mutation in individual LFS families need to be established.

We report here the phenotypic abnormalities of EBV-transformed LCLs of LFS patients with various germ-line p53 gene mutations. Abnormalities could consistently be seen in the control of apoptosis and in several phases of cell cycle regulation, suggesting that these findings are significant and relevant to the susceptibility of LFS families to genomic instability and cancer at an early stage of their lives. We propose that the approach presented here is a potentially useful method not only for understanding the cancer susceptibility of these families on the basis of their germ-line p53 mutation, but for the individual screening of LFS families as well.

MATERIALS AND METHODS

EBV-transformed LCLs. PBLs were obtained from donors and separated by Ficoll-Hypaque gradient centrifugation. EBV-immortalized LCLs were established by infecting PBLs from normal individuals and symptomatic carriers with the EBV strain B95–8 as described previously (18, 19). The samples were obtained after obtaining the informed consent of the donors and the approval of the local committee for ethics at the National Children's Hospital, Tokyo.

Western and Northern Blot Analyses. LCLs were harvested before and 6 and 12 h after 5 Gy of γ-irradiation. mRNA was purified from total RNA (70 μg) using 40 μl of Dynabeads oligo(dT)<sub>25</sub> (Nihon Dynal, Tokyo, Japan).

3 The abbreviations used are: mt, mutant; wt, wild-type; LCL, lymphoblastoid cell line; LFS, Li-Fraumeni syndrome; PBL, peripheral blood lymphocyte; PCNA, proliferating cell nuclear antigen; TNF, tumor necrosis factor; FASAY, functional analysis of separated alleles in yeast; RT-PCR, reverse transcription-PCR; PI, propidium iodide; FISH, fluorescence in situ hybridization; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; CDK, cyclin-dependent kinase; PB, peripheral blood; MNC, mononuclear cell.

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2 Present address: Department of Pediatrics, Yamanashi Medical University, 1110 Shimokawaguchiko, Daito-cho, Nakakama-Gun, Yamanashi 409-38, Japan.

3 Present address: The Second Department of Internal Medicine, Nagoya City University Medical School, 1 Kawasaki, Mizuho-cho, Mizuho-ku, Nagoya 467, Japan.

4 To whom requests for reprints should be addressed. Phone: 03-3414-8121; Fax: 03-3419-4757; E-mail: smizutani@nch.go.jp.

5 The abbreviations used are: mt, mutant; wt, wild-type; LCL, lymphoblastoid cell line; LFS, Li-Fraumeni syndrome; PBL, peripheral blood lymphocyte; PCNA, proliferating cell nuclear antigen; TNF, tumor necrosis factor; FASAY, functional analysis of separated alleles in yeast; RT-PCR, reverse transcription-PCR; PI, propidium iodide; FISH, fluorescence in situ hybridization; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; CDK, cyclin-dependent kinase; PB, peripheral blood; MNC, mononuclear cell.
followed by electrophoresis in 0.9% Seakem agarose gel (FMC Bioproducts, Rockland, ME). p21<sup>3p-1/wat-1</sup> and β2MG probes were synthesized by PCR amplification of cDNA using the primers 5'-GGCCCATGTCGACCGG-3' (forward) and 5'-CACAATCTCGAATGAGCA-3' (reverse) for p21<sup>3p-1/wat-1</sup> and the primers 5'-ACCCACTGGAAGAGATGA-3' (forward) and 5'-ATCCTCAAATCCTCAGAT-3' (reverse) for β2MG. Radioactivity for the hybridizing signals in Northern blot membrane was analyzed using a Fuji BAS2000. Soluble total cellular protein was electrophoresed in Multigel 10 (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan) and transferred to an Immobilon polyvinylidene difluoride transfer membrane (Daichi Pure Chemicals Co., Ltd.). Binding of the primary antibody was detected using a commercial enhanced chemiluminescence kit (Amersham Japan, Tokyo, Japan). Antibodies to p53 (AB2, α-tubulin, and PCNA were obtained from Oncogene Science (Cambrige MA). Antibodies to cyclin E (reactive with D1 and D2) and CDK2 were obtained from Zymed, San Francisco, CA. To determine whether EBV infection might change the expression pattern of wt versus mt p53 in LCLs and precultured PB MNCs from patients with a germ-line p53 mutation, EBV-immortalized LCLs were established from the PBLs of symptomatic patients. Three LCLs from each family were analyzed before and after EBV immortalization in three patients by single-strand conformational polymorphism analysis of RT-PCR products, which were amplified by PCR using primers 5'-ATCTGCTCAAATCCTCAGAT-3' and 5'-ATCTGCTCAAATCCTCAGAT-3' (reverse) for [γ-<sup>32</sup>P]ATP. Samples were electrophoresed in Multigel 15/25 (Daichi Pure Chemicals). The relative increase was presented as 
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\frac{([\text{kinase activity with irradiation}]) - ([\text{kinase activity without irradiation}])}{([\text{kinase activity without irradiation}])} \times 100\%.
\]

**RESULTS**

**Clinical Description.** Family 1 had a p53 germ-line missense mutation at residue 286 (GAA to GCA), as has been reported previously (24). In family 2, the proband’s germ-line alteration was a missense mutation at residue 133 (ATG to AGG). She developed unilateral and contralateral breast cancer at the ages of 26 and 27, respectively. Her mother died of a brain tumor at the age of 43. Two of the proband’s brothers and one of her sisters developed lymphoma, brain tumor, and bilateral breast cancer, respectively. In family 3, the germ-line abnormality was a missense mutation at residue 282 (CGG to TGG), as reported by Shiseki et al. (25). The proband developed osteogenic sarcoma, stomal sarcoma of the breast, and gastric carcinoma at the ages of 13, 19, and 22, respectively. Because her mother did not show any germ-line p53 mutation, it is most likely that her father was the affected patient carrying the germ-line mutation, although he could not be examined. Family 4 had a germ-line missense mutation at residue 132 (AAG to GAG). The 15-year-old proband was diagnosed as having rhabdomyosarcoma at the age of 2 and osteosarcoma at the age of 11. His mother and maternal grandmother both died of breast cancer. The proband’s elder brother (34 years old) had the same germ-line p53 mutation. Family 5 was characterized by a nonsense mutation at residue 213 (CCG to TGA), as reported by Shiseki et al. (25). The proband acquired stomach cancer at the age of 41 and lung cancer a year later (17). The details of families 1, 3, and 5 have been reported previously (17, 24, 25).

**Expression Pattern of wt Versus mt p53 in LCLs and Precultured PB MNCs from Patients with a Germ-line p53 Mutation.**

EBV-immortalized LCLs were established from the PBLs of symptomatic carriers of p53 germ-line mutations (286A, 133R, 282W, 132E, and 213ter LCLs from families 1, 2, 3, 4, and 5, respectively). Single-strand conformational polymorphism analysis of RT-PCR products showed that both the wt and mt p53 transcripts were expressed in 286A, 133R, 282W, and 132E LCLs (data not shown). In 213ter LCL, the expression of mt p53 was barely detectable (17). These data were confirmed by a FASAY (21), as described below. To determine whether EBV infection might change the expression pattern of wt and mt p53 alleles, the levels of these transcripts were analyzed before and after EBV immortalization in three patients by...
PCR-single-strand conformational polymorphism. The wt:mt p53 mRNA ratio in 133R and 282W LCLs was comparable to that found in fresh PB MNCs of the corresponding patient, even after 2 years of in vitro culture (data not shown). Predominant expression of wt p53 was seen in 213ter LCL after 1 year of in vitro culture, and this was identical to that found in precultured PB MNCs, as reported previously (17).

Transactivation Activity of p53 Protein Encoded by Separated Alleles in LFS-derived LCLs. To clarify the functional activity of these mutations, we used an assay based on the FASAY, which allows the determination of the transactivating property of each mt species of p53 under homozygous conditions. As a result, 286A, 133R, 282W, and 132E LCLs were found to have 48, 41, 40, and 29% His+ colonies, respectively, among the total colonies analyzed (Table 1), indicating that these mutations are transcriptionally inactive when homozygous. This result also indicates that approximately 30–50% of p53 transcripts were derived from the mt p53 allele in these LCLs. These findings were in contrast to the 98–100% His+ colonies observed in normal controls and the 88% His+ colonies observed in 213ter LCL. The level seen in the latter is consistent with the finding that the expression of wt p53 predominated over that of mt p53 in 213ter LCL (17).

p21cip-1/waf-1 Induction Is Variably Dysregulated in LFS-derived LCLs. p21cip-1/waf-1 is the major inhibitor of the activities of cyclin/CDK/PCNA complexes and is transactivated by p53 in response to DNA-damaging agents. Accordingly, we studied the p53-induced expression of p21cip-1/waf-1 6 and 12 h after 5 Gy of γ-irradiation in these LFS-derived LCLs. p21cip-1/waf-1 protein was found to be barely detectable in any of the untreated LCLs. After irradiation, a remarkable increase in p21cip-1/waf-1 protein was seen in normal LCLs. On the other hand, the induction of p21cip-1/waf-1 protein in irradiated 286A, 133R, and 132E LCLs remained significantly low; levels were less than 50% of that seen in irradiated normal LCL throughout the 12-h observation period. In 282W and 213ter LCLs, p21cip-1/waf-1 protein was transactivated by up to 80 and 50%, respectively, of that derived LCLs. p21cip-1/waf-1 is the major inhibitor of the activities of cyclin/CDK/PCNA complexes and is transactivated by p53 in response to DNA-damaging agents. Accordingly, we studied the p53-induced expression of p21cip-1/waf-1 6 and 12 h after 5 Gy of γ-irradiation in these LFS-derived LCLs. p21cip-1/waf-1 protein was found to be barely detectable in any of the untreated LCLs. After irradiation, a remarkable increase in p21cip-1/waf-1 protein was seen in normal LCLs. These findings were supported by Northern blotting of 286A, 133R, and 282W LCLs 6 h after irradiation. p21cip-1/waf-1 mRNA increased markedly in normal LCLs. Although 282W LCL showed slightly less enhanced expression, it was still significantly higher than that seen in 286A and 133R LCLs (Fig. 1B). The leukemic cell lines HL-60 and KOPM28 (26), with null p53 expression, respectively, among the total colonies analyzed (Table 1), indicating that these mutations are transcriptionally inactive when homozygous. This result also indicates that approximately 30–50% of p53 transcripts were derived from the mt p53 allele in these LCLs.

Table 1 Functional assay of mt p53 for separated allele in yeast in LFS-derived LCLs

<table>
<thead>
<tr>
<th>LCL</th>
<th>His+ / total colonies analyzed (%)</th>
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<tbody>
<tr>
<td>wt/wt</td>
<td>98 – 100</td>
</tr>
<tr>
<td>286A</td>
<td>48</td>
</tr>
<tr>
<td>133R</td>
<td>41</td>
</tr>
<tr>
<td>282W</td>
<td>40</td>
</tr>
<tr>
<td>132E</td>
<td>29</td>
</tr>
<tr>
<td>213ter</td>
<td>88</td>
</tr>
</tbody>
</table>

This table shows that the expression of wt p53 predominated over that of mt p53 in 213ter LCL (17).

Activity in LFS-derived LCLS after γ-Irradiation. Cyclin/CDK2 and cyclin A/CDK2 complexes have been shown to be involved in our LCLs and is consistent with the results observed in other leukemic cell lines (Refs. 27 and 28; data not shown). These results indicate that the level of p21cip-1/waf-1 induction after DNA damage is impaired to a variable degree, depending on the nature of the p53 mutation in the LFS-derived LCLs.

Cell Cycle Dysregulation in LFS-derived LCLs in Response to γ-Irradiation. It is well known that cell cycle arrest in G1, in response to DNA damage is mediated by p53-induced p21cip-1/waf-1 transactivation. To assess p53 function, G1 growth arrest induction was examined in these LCLs 16 h after 5 Gy of γ-irradiation. The LCLs with 286A, 133R, 132E, and 213ter mutations showed a slowdown of G2-G1 arrest induction (the percentage of increase in G0-G1 was −3.9 ± 7.3, −6.6 ± 7.5, −4.0 ± 0.29, and 1.2 ± 6.1%, respectively; Fig. 2). In contrast, 282W LCL showed a significant increase in the G2-G1 fraction (percentage of increase, 7.4 ± 10.0%) after irradiation up to a level that was not significantly different from that seen in normal LCL (percentage of increase, 10.5 ± 7.6%). To confirm this unexpected feature of the 282W mutation, another clone, 282W LCL', was established from the elder sister of the proband in family 3. 282W LCL' also showed a significant increase in the G2-G1 fraction after irradiation (data not shown). This supports our finding that the 282W mutation is unique among the heterozygous mutations with regard to cell cycle control. The abnormalities in G1 cell cycle regulation in 286A, 133R, 132E, and 213ter LCLs are consistent with defective p21cip-1/waf-1 induction in these LCLs, which most likely results from the dominant negative transactivation activity of mt p53 species when heterozygous.

Abnormal Inhibition of Cyclin E- and A-associated Kinase Activity in LFS-derived LCLs after γ-Irradiation. Cyclin E/CDK2 and cyclin A/CDK2 complexes have been shown to be...
essential for the G1 to S-phase and S-phase to G2 transition, respectively, and both of them are subject to inhibition by p21cip-1/waf-1 in response to DNA-damaging agents. As reported above, 286A, 133R, 132E, and 213ter LCLs but not 282W LCL showed dysregulation of the G1-S-phase checkpoint associated with defective p21cip-1/waf-1 transactivation after γ-irradiation. These observations prompted us to investigate the histone H1 kinase activity associated with cyclin/CDK complexes (Fig. 3). Inhibition of cyclin E/CDK2-associated histone H1 kinase activity was significantly reduced in 286A, 133R, 132E, and 213ter LCLs, but not in 282W LCL (Fig. 3A). The substantial inhibition of cyclin E-associated kinase activity in 282W LCL was consistent with normal radiation-induced G1 arrest. In contrast, the inhibition of cyclin A-associated histone H1 kinase activity was not sufficient in any of the LFS-derived LCLs, including 282W LCL (Fig. 3B), suggesting that control of S-phase-G2 is disrupted in these LCLs.

To rule out the possibility that the abnormal cell cycle kinetics and the change in the biochemical characteristics of cyclin/CDK complexes only reflected the level of cyclin E or A, the expression of these cyclins was analyzed by Western blotting. No significant difference in the expression of these cyclins or in that of CDK4 and PCNA was found in LCLs carrying either the wt/wt or wt/mt p53 when the cells were examined 16 h after γ-irradiation (data not shown).

Defect in Control of the Mitotic Checkpoint in LFS-derived LCLs. p53 has been implicated not only in a G1 checkpoint, but also in the later phases of the cell cycle, such as PCNA-dependent DNA replication and the mitotic checkpoint, both of which are essential for the maintenance of diploidy. To investigate a possible abnormality in the mitotic checkpoint in LFS-derived LCLs, in 286A and 282W LCLs, which showed 20 and 80% of the p21cip-1/waf-1 transactivation of a normal LCL, respectively, the proportion of the cells that developed a second round of DNA synthesis without cytokinesis after 72 and 144 h of exposure to 5 Gy of γ-irradiation was examined. Characteristic phenotypes such as decreased apoptosis (shown later), moderate G2 delay, and accumulation of DNA contents greater than 4C were seen in both of the heterozygous LCLs (Fig. 4A). The 8C cell population started to accumulate at 72 h and continued to increase until 144 h after irradiation. There was a notable difference in the proportion of octaploid cells between normal LCL (less than 1.0%) and LCLs carrying the 286A (11.0%) or 282W (9.0%) mutation at 144 h after irradiation (Table 3). These findings were also confirmed by FISH using centromere probes for chromosomes 1, 11, and 16 (Fig. 4B).

These results indicate that LFS-derived LCLs consistently show significant defects in the mitotic/spindle checkpoint and that they are highly susceptible to genomic instability in response to a genotoxic substance.

Radiation-induced Cell Death Is Dysregulated in LFS-derived LCLs. Cells from mice deficient in wt p53 display a reduced sensitivity to DNA-damaging agents and an increased failure to enter apoptosis (29). To determine whether p53 heterozygous mutations confer resistance against cell death stimuli, LFS-derived LCLs were irradiated with 5 Gy and examined for apoptotic cells 24 and 48 h later. At 24 h, the percentage of the subdiploid fraction was slightly greater in normal than in LFS-derived LCLs, and this difference was significantly increased at 48 h, with 286A, 133R, 132E, and 213ter LCLs, respectively (Table 2). 282W LCL, despite exhibiting an apparently normal G1-G2 arrest, was nonetheless modestly resistant to radiation-induced apoptosis (Table 2 and Fig. 4). One of the data in normal, 286A, and 282W LCLs 48 h after irradiation was further verified by the TUNEL method, and 53, 16.3, and 13.2% of the cells were shown to be in the apoptotic population, respectively (data not shown). All these data suggest that the induction of apoptosis is consistently impaired in LCLs with various types of germ-line p53 mutation.

DISCUSSION

To study the biological behaviors of LFS-derived LCLs heterozygous for p53 gene mutations, control of the cell cycle and apoptotic cell death in response to γ-irradiation were investigated. LCLs were
Table 2. Increase in the apoptotic cell population before irradiation (0 h) and 24 and 48 h after 5 Gy of irradiation in LFS-derived LCLs.

The apoptotic cell population was measured by FACScan as described in the text. The values are the means ± SD (n > 3 for each genotype).

<table>
<thead>
<tr>
<th>LCL</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
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<tbody>
<tr>
<td>wt/wt</td>
<td>2.07 ± 0.76</td>
<td>11.98 ± 4.96</td>
<td>28.63 ± 3.28</td>
</tr>
<tr>
<td>286A</td>
<td>2.68 ± 1.85</td>
<td>5.52 ± 1.53&quot;</td>
<td>9.56 ± 1.37&quot;</td>
</tr>
<tr>
<td>133R</td>
<td>2.92 ± 1.24</td>
<td>5.49 ± 1.59&quot;</td>
<td>9.47 ± 1.90&quot;</td>
</tr>
<tr>
<td>282W</td>
<td>2.63 ± 1.89</td>
<td>5.03 ± 1.05&quot;</td>
<td>13.62 ± 3.99&quot;</td>
</tr>
<tr>
<td>132E</td>
<td>1.85 ± 1.68</td>
<td>2.54 ± 1.30&quot;</td>
<td>5.17 ± 2.15&quot;</td>
</tr>
<tr>
<td>213ter</td>
<td>2.67 ± 1.31</td>
<td>5.16 ± 2.28&quot;</td>
<td>7.20 ± 5.39&quot;</td>
</tr>
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*p < 0.05.

IR (-) (+)

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established from symptomatic carriers representative of the five LFS families showing different constitutional p53 mutations. EBV-immortalized LCLs maintain expression of both wt and mt p53 alleles during long-term in vitro culture, as demonstrated by RT-PCR and FASAY (in this paper and Ref. 18), possibly due to partial transformation by EBV-encoded EBV nuclear antigens and latent membrane protein-1. Although EBV nuclear antigen 5 and BZLF1 have been reported to bind to p53 protein (30, 31), this interaction seems to be negligible with regard to p53-dependent transactivation and cell cycle control, at least in in vitro transformed LCLs (32). We and others have seen that the relative level of wt versus mt p53 mRNA varies among families with germ-line p53 gene mutations (15–17). The expression pattern of wt versus mt p53 in precultured PB MNCs has been preserved in LCLs from families 2, 3, and 5 (data not shown). These findings indicate that we can investigate the functional properties of each mt p53 in a heterozygous context using EBV-immortalized LCLs.

p53 is a tumor suppressor gene with pleiotropic functions that include the control of genomic plasticity and integrity. Transactivation of p21<sup>cip-1/waf-1</sup> by p53 seems to be indispensable in these processes. Although the function of p21<sup>cip-1/waf-1</sup> has not been fully elucidated, inhibition of the kinase activity of multiple cyclin/CDK complexes has been shown to play a central role in cell cycle regulation. DNA damage-induced inhibition of cyclin E-associated kinase activity was absent in 286A, 133R, 132E, and 213ter LCLs, but not in 282W LCL. These findings are consistent with the results of cell cycle analyses showing that 286A, 133R, 132E, and 213ter LCLs fail to arrest in G1 after irradiation, whereas 282W LCL was significantly blocked in G1. On the other hand, the inhibition of cyclin A-associated kinase activity was insufficient in all of the heterozygous clones, including 282W
CELL CYCLE AND APOPTOSIS CONTROL IN CELLS WITH p53 MUTATION

Fig. 4. A, flow cytometric analysis of the mitotic checkpoint in LFS-derived LCLS. LCLs before (0h), 72 h (72h), and 144 h (144h) after irradiation were analyzed with regard to their DNA contents by flow cytometry as described in “Materials and Methods.” Two heterozygous LCLs (286A and 282W) with different transactivation activity of p21cip-1/waf-1 and wt/wt LCL are shown. B, FISH signals in a polyploid cell seen in 286A LCL 144 h after γ-irradiation. FISH signals are seen as white dots, and nuclear morphology is revealed by PI staining. FISH analysis showed 20% of the treated cells contained more than 4 copies of chromosome 1, 11, and 16. An example using D125 probe for chromosome 1 is shown.

LCL. On the basis of these findings, all of the LFS-derived LCLs seem to have a defect in the control of S-phase-G2 in response to DNA-damaging agents, suggesting that this defect may universally underlie the susceptibility of LFS patients to cancer. These findings, along with the p21cip-1/waf-1 transactivation data, also suggest that although inhibition of cyclin E/CDK2 kinase can be achieved by a certain threshold concentration of p21cip-1/waf-1, inhibition of cyclin A/CDK2-associated kinase activity may require an even higher threshold level of p21cip-1/waf-1. These interpretations are supported by the previous observation that cyclin E/CDK2 has a higher affinity for
p21cip-1/waf-1 binding (33). Thus, differences in the effective threshold concentration of p21cip-1/waf-1 after irradiation may explain the phenotypic diversities of cell cycle control in these LCLs.

The apparently insufficient transactivation of p21cip-1/waf-1 suggests that the defect in cell cycle control in LFS-derived LCLs is not limited to G1 or S-phase-G2 phase. The mitotic checkpoint has also been shown to be under the control of p53 (34). This finding was further extended by Waldman et al. (35), who showed that S-phase-M-phase coupling in response to a DNA-damaging agent is controlled by p21cip-1/waf-1. Two representative LFS-derived LCLs, 286A and 282W, with minimum and maximum p21cip-1/waf-1 transactivation activity were studied with respect to the proportion of cells with DNA contents greater than 4C. The results showed that both of these LCLs were associated with a significant accumulation of polyploid cells with DNA contents exceeding 4C. This indicates that the deregulation of the mitotic/spindle checkpoint is a common biological abnormality in LFS-derived LCLs. Thus, in the presence of a DNA-damaging agent, there seems to be a variable abrogation of G1 arrest and consistent dysregulation of the S-phase-G2 phase and mitotic checkpoint in LFS-derived LCLs.

In all of these LCLs, a defect in the induction of apoptosis in response to γ-irradiation was seen by PI staining, part of which was further verified by the TUNEL method. In the light of results showing that p21cip-1/waf-1-deficient mice possess a normal response with respect to apoptosis (36), the defect that we see in apoptosis is difficult to account for on the basis of a poor response in p21cip-1/waf-1 transactivation. Rather, these findings support the hypothesis that p53-dependent activation for apoptosis requires different target genes (37). Alternatively, the inhibition of apoptosis could be directly induced by the mt allele of the p53 gene in the form of a gain of function effect in 286A, 133R, 282W, and 132E LCLs or in a manner that relies on the level of wt p53 function in 213ter LCL. The expression of proteins of the bcl-2 gene family was analyzed by Western blotting to determine their relevance to the observed failure of LCLs to enter apoptosis. Bcl-2 and bax proteins are of particular interest, because their transcription is reciprocally regulated in cells undergoing apoptosis, apparently by the interaction of p53 with regulatory DNA sequences present in their genes (5). The steady-state levels of bcl-2 and bax proteins, however, remained unchanged during the 48-h period after irradiation in these clones, including normal LCLs, suggesting that these genes do not directly mediate apoptosis in our cells. It should be noted, however, that p53 may trigger apoptosis in the case of other mutations. This observation that the 282W mutation abrogates the sequence-specific DNA binding capacity (39, 40). These findings may suggest that mt p53 species, which are inactive with regard to transactivation in a homopolymerized context, can variably interfere with wt p53 function in a heterozygous context. Our findings point to the need for a study of the biological features of cells from carriers with individual germ-line p53 mutations.

The results presented here argue against a recent report showing that LCLs with 257Q, 342ter, and 257FS heterozygous mutations maintain p53 function during long-term in vitro culture (18). Although their study did not include an analysis of the mitotic checkpoint, it is likely that the apparently normal characteristics of those LCLs correspond to those seen in 282W LCLs, suggesting that these mutations will also show mt p53 activities in the control of the later phases of the cell cycle and apoptosis when analyzed in the presence of a DNA-damaging agent.

In summary, although the impairment of G1 cell cycle control is variable, dysfunctions in the control of apoptosis and cell cycle control at S-phase-G2 and the mitotic checkpoint are consistently seen in LFS-derived LCLs in response to DNA-damaging agents, thus suggesting that these dysfunctions underlie, at least in part, the susceptibility of LFS families to cancer. Furthermore, the approach presented here is a potentially useful method for studying individual carriers of different germ-line p53 mutations and different biological features.

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REFERENCES


D. Delia and S. Mizutani, unpublished observations.


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Kumiko Goi, Masatoshi Takagi, Satoshi Iwata, et al.


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