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Original Article

Different Responses to 5-fluoraouracil in Mutagenicity and Gene Expression between Two Human Lymphoblastoid Cell Lines with or without *TP53* Mutation

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Human lymphoblastoid TK6 and WTK-1 cells are widely used to detect mutagens *in vitro*. TK6 cells have wild-type *TP53* alleles, while WTK-1 cells have one allele of mutated *TP53*. Both cells were treated with 5-fluorouracil (5-FU), and gene mutation assay and micronucleus assay were performed to clarify the differential response related to the *TP53* gene status. The effects of 5-FU on gene expression were assessed by microarray and quantitative RT-PCR analyses. In WTK-1 cells, 5-FU increased the frequency of cells with micronucleus and mutation. In TK6 cells, frequency of cells with micronucleus was increased but the mutation frequency was not. The cytotoxicity induced by 5-FU was more prominent in TK6 cells than in WTK-1 cells. Analysis of gene expression showed that the genes involved in the *TP53* pathway were up-regulated in TK6 cells but not in WTK-1 cells. The differential responses to 5-FU between these cell lines appeared to be due to the difference in the *TP53* gene status, thus providing a molecular basis for the bioassays using these cell lines in the toxicology field. Our results indicate that the clinical efficacy of 5-FU chemotherapy may depend on the *TP53* genotype.

Key words: 5-fluorouracil, *TP53*, *Tk* mutation assays, microarray analysis

In the toxicology field, there are many ways to assess the adverse effects of poisons on humans. Gene mutation assays (*Tk* mutation assays) and *in vitro* micronucleus assays detect DNA damage and chromosomal aberration, respectively. These studies are performed as screening tests for carcinogenicity. Human lymphoblastoid TK6 cells and WTK-1 cells are widely used in *Tk* mutation assays or *in vitro* micronucleus assays. These cells are heterozygous at the *Tk* locus, where loss of heterozygosity (LOH) by DNA damage leads to the detection of mutants by resistance to the selective agent (trifluorothymidine) [1–4].

TK6 and WTK-1 cells are both derived from WIL-2 cells that were originally derived from a 5-year-old male with hereditary spherocytosis and were transformed by the Epstein-Barr virus [5]. TK6 and WTK-1 cells are not classified as lymphoma or leukemia, and there is no data indicating that these cell lines have tumorigenic activity *in vivo*. TK6 cells have wild-type *TP53* tumor suppressor genes, while WTK-1 cells have a mutant *TP53* gene [6]. The *TP53* gene in WTK-1 cells is mutated at codon 237 of exon 7, which

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is the part of the central DNA binding domain [6]. TK6 and WTK-1 cells are useful for examining the function of *TP53* status, because these cells have an almost identical genetic background except for *TP53* status.

TP53 protein is involved in DNA repair, G_1 checkpoint activation, and induction of apoptosis [7]. Wild-type TP53 leads cells with reparable DNA damage into cell cycle arrest, allowing repair of the damage, and cells with severe DNA damage to apoptosis, although mutated TP53 does not. The expression of genes related to apoptosis, the cell cycle, and DNA repair was up-regulated in TK6 cells than in WTK-1 cells after treatment of direct DNA damaging agents and irradiation of X-ray [8, 9].

Earlier studies have shown that, compared with *TP53* mutant or null cells, *TP53* wild-type cells undergo apoptosis earlier or at a higher frequency following severe cytotoxicity induced by X-rays or certain chemicals [3, 6, 10–13]. *TP53* mutant cells differ from the wild-type cells in their cytotoxic and mutagenic responses to some mutagens [14–17]. Some clastogens and spindle poisons are more cytotoxic in wild-type cells than in mutant cells and, at equal levels of cytotoxicity, are more clastogenic in the mutant cells [3, 18]. The spontaneous mutation frequency in WTK-1 cells is $100 - 200 \times 10^{-6}$, while it is less than 10×10^{-6} in TK6 cells [14]. WTK-1 cells are more resistant than are TK6 cells to X-rays and other mutagens [14]. These differences may be due to differences in *TP53* status.

5-Fluorouracil (5-FU), a pyrimidine base analog, competitively inhibits DNA synthesis via the inhibition of thymidylate synthetase (TS) [19–21]. 5-FU is clastogenic in mammalian cells [22] *in vitro* and *in vivo* [23] but is not mutagenic to *Salmonella typhimurium* [22]. 5-FU induces neither chromosomal aberrations nor sister chromatid exchanges in the human materials studied so far [24]. However, it was not clear that the difference in status of *p53* in human materials is related to the degree of DNA damage induced by 5-FU. In addition, the carcinogenic activity of 5-FU has not yet been fully assessed in clinical trials.

In our previous report [25], 3 h treatment with 5-FU increased mutation frequency in mouse lymphoma cells but not in TK6 or WTK-1 cells. However, 5-FU was known to inhibit the DNA synthesis more successfully in longer-term treatment than in short-term treatment [26], and 5-FU and its related drugs are used frequently in long-term administration in clinical trials.

We aimed to determine the potential of 5-FU for mutagenicity after long-term (24 h) treatment in human cell lines. Furthermore, the gene expression profile after 5-FU treatment was analyzed to examine whether there was a differential response between these cell lines compared with the direct DNA damaging agents such as alkylating agents or X-ray.

Materials and Methods

Chemical and cell lines. 5-FU (CAS No. 51–21–8) was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan) and dissolved in physiologic saline just before use.

TK6 and WTK-1 cells were provided by Hatano Research Institute at the Food and Drug Safety Center. The cells were cultured in RPMI 1640 medium (Gibco, Invitrogen Corp., Carlsbad, CA, USA) containing 10 v/v% horse serum (Gibco, Invitrogen Corp.) at 37℃ in an atmosphere of 5% CO_2 and high humidity.

Mutation analysis of the TP53 gene. Genomic DNA was isolated from the cell lines by SDS/proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. Coding exons of the *TP53* gene were amplified with the intronic primers as follows: sense 5'-GAC TGC TCT TTT CAC CCA TCT A and antisense 5'-TGC AGG GGG ATA CGG CCA G for exon 4, sense 5'-TTT GCT GCC GTG TTC CAG TTG and antisense 5'-ACC CTT AAC CCC TCC TCC CA for exon 5–6, sense 5'-CTT GGG CCT GTG TTA TCT CCT and antisense 5'-CGC CGG AAA TGT GAT GTG AGG for exon 7, sense 5'-CCT TAC TGC CTC TTG CTT CTC and antisense 5'-GAT AAG AGG TCC CAA GAC TTA G for exon 8–9. PCR products were purified with exonuclease I and shrimp alkaline phosphatase (USB Corporation, Cleveland, OH, USA), reacted with a Big Dye Terminator (Applied Biosystems, Foster City, CA, USA), and analyzed on an ABI 3130 sequencer (Applied Biosystems).

Tk mutation assay. Considering its cytotoxicity, the dose levels of 5-FU were selected as 0.125, 0.25, 0.5, and 1.0 μg/mL for 24 h in TK6 and WTK-1 cells in *Tk* mutation assays. The *Tk* mutation assays

were performed by the microwell method [14]. Namely, the cells (2×10^6 cells, cell density at 2×10^5/mL) were treated at 37℃ for 24 h and washed. A portion of the cells was seeded onto 96-well plates, and the plating efficiency (PE) was measured on day 0 after 5-FU treatment (PE0). The remaining cells were cultured for 72 h to allow phenotypic expression. The cells were seeded onto 96-well plates in the presence of trifluorothymidine (TFT, CAS No. 70-00-8, Sigma Chemical Co., St. Louis, MO, USA) for assessment of mutation frequency. Another portion of the cells was seeded onto 96-well plates in the absence of TFT for the measurement of plating efficiency after the expression period.

The plates were incubated for 12-14 days, and the number of wells containing colonies was then counted, allowing us to evaluate the mutation frequency [14]. Mutation frequency and relative cell survival from PE0 were calculated as previously described [27].

In vitro micronucleus assay. The cells were treated by 5-FU in the same manner as in the *Tk* mutation assay. The micronucleus (MN) assay specimens were prepared 48 h after 5-FU treatment, as previously described [28]. Briefly, 10^6 to 3×10^6 cells were treated with hypotonic (75 mM) KCl solution, fixed in methanol-acetic acid (1 : 3), resuspended in methanol containing 1% (v/v) acetic acid, and placed onto glass slides in sample drops. The specimens were stained with acridine orange (Wako Pure Chemicals Industries, Ltd.) and were observed with the aid of a fluorescence microscope with a 470-490 nm excitation filter. One thousand cells from each treatment group were observed, and we counted the number of cells with MN. Cells were considered to be positive for MN when the diameter of MN was less than half the diameter of the main nucleus. We excluded cells with an abnormal main nucleus from this analysis.

Analysis of gene expression by microarray. The gene expression after 1 μg/mL of 5-FU treatment for 24 h was analyzed by Agilent Microarray. Total RNA was extracted from $0.2-6 \times 10^6$ cells after 5-FU treatment. Total RNA was prepared using RNeasy Mini kit (Qiagen, Inc., Hilden, Germany) according to the manufacturer's instructions. RNA samples were amplified and Cy3-labeled using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's instructions. The hybridization was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). Briefly, 1.65 μg Cy3-labeled fragmented cRNA was hybridized overnight (17 h, 65℃) to Agilent Whole Human Genome Oligo Microarrays 4 × 44K. Fluorescence signals of the hybridized microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies). The Agilent Feature Extraction Software was used to read out and process the microarray image files.

The signal intensities were normalized by dividing the intensity values by their median. The genes with considerable change of expression, more than 2-fold and with a *p*-value less than 0.01 compared with the solvent control, were selected by the Resolver® Software (Agilent Technologies). The analysis of function of the genes with changed expression after 5-FU treatment was done by DAVID bioinformatics resources ver. 6.7.

Analysis of gene expression by quantitative RT-PCR. The gene expression after 1 μg/mL of 5-FU treatment for 24 h was analyzed by TaqMan® Gene Expression Assays. Total RNA was extracted in the same manner as the analysis by microarray. Total RNA was reverse-transcribed by High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) according to the manufacturer's instructions. TaqMan® Gene Expression Master Mix (Applied Biosystems) was used for quantitative RT-PCR, and 20 ng of cDNA was used as the template. TaqMan® Gene Expression Assays (Applied Biosystems) with assay IDs Hs00180269_m1, Hs00355782_m1, Hs00540450_s1, and Hs00266705_g1 were used for analysis of *Bax*, *p21*, *MDM2*, and *GAPDH*, respectively.

PCR conditions consisted of one cycle at 50℃ for 2 min followed by 95℃ for 10 min and 40 cycles of 2 segments (95℃ for 15 s, 60℃ for 1 min) using ABI 7500 realtime PCR system (Applied Biosystems). Each sample was normalized using *GAPDH* as a reference for overall expression level.

Statistical analysis. When the mutation frequency increased significantly compared with the vehicle control by Dunnett's test, 5-FU treatment was judged to be positive in *Tk* mutation assay. In the analysis of cytotoxicity (PE0), the number of wells

containing colonies was compared with the vehicle control by chi-square test. The conditional binomial test was performed to judge the positive response in MN frequency. The gene expression by Quantitative RT-PC was analyzed by Student's *t*-test. SAS (system release 8.2) or Microsoft Excel was used for statistical analysis. *P* values <0.05 were taken as significant.

Results

Tk mutation assay and in vitro micronucleus assay. Initially, the *TP53* gene of WTK-1 and TK6 cells was analyzed by DNA sequencing, and it was confirmed that methionine (ATG) was substituted by isoleucine (ATA) at codon 237 in exon 7 of the *TP53* gene in WTK-1 cells (Fig. 1A).

5-FU was cytotoxic in both cell lines in a concentration-dependent manner (Fig. 1B). The cell survival

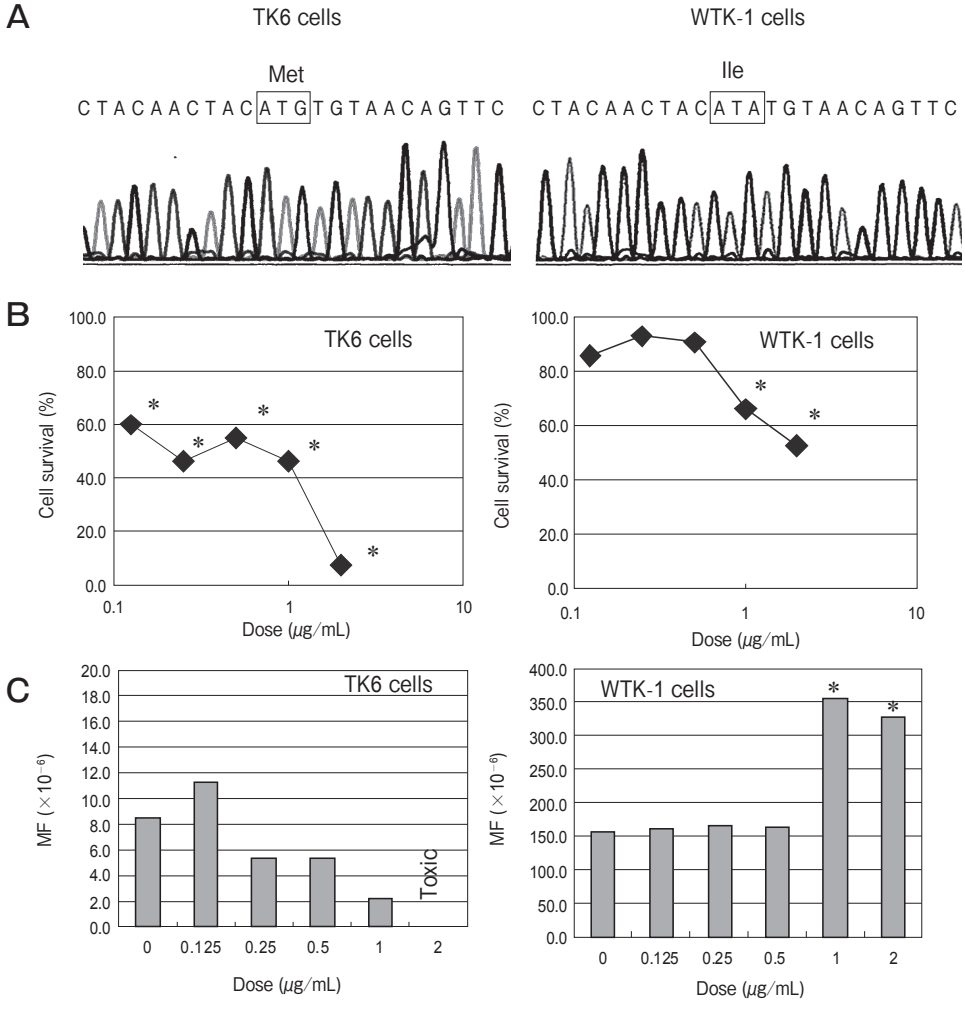


Fig. 1 *TP53* mutation, cytotoxicity, and mutation frequency of TK6 and WTK-1 cells. Sequencing results of exon 7 in the *TP53* gene (**A**), cell survival (**B**), and mutation frequency after 24 h treatment with 5-FU (**C**), in TK6 (*left*) and WTK-1 cells (*right*). MF, mutation frequency. The *TP53* gene in TK6 cells has no mutation; however, the *TP53* gene in WTK-1 cells has the substitution of isoleucine (ATA) for methionine (ATG) at codon 237 in exon 7 (**A**). The concentration-dependent cytotoxicity was shown in TK6 and WTK-1 cells after 5-FU treatment and was more severe in TK6 cells than in WTK-1 cells (**B**). 5-FU increased MF about 2-fold relative to the solvent control at 1.0 μg/mL or more In WTK-1 cells but not in TK6 cells (**C**).
*significant difference ($p < 0.05$) from the vehicle control by chi-square test (**B**) and Dunnett's test (**C**). Note that the scale of MF in WTK-1 cells is 25-fold higher than that inTK6 cells.

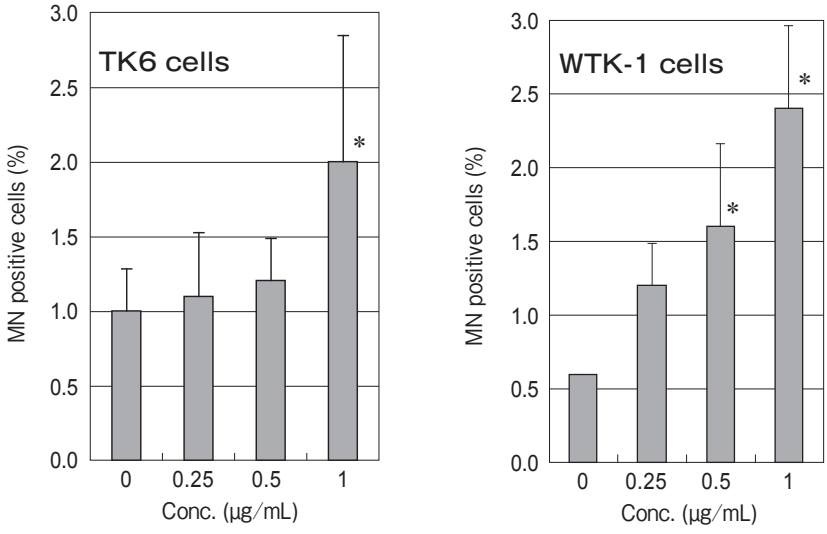


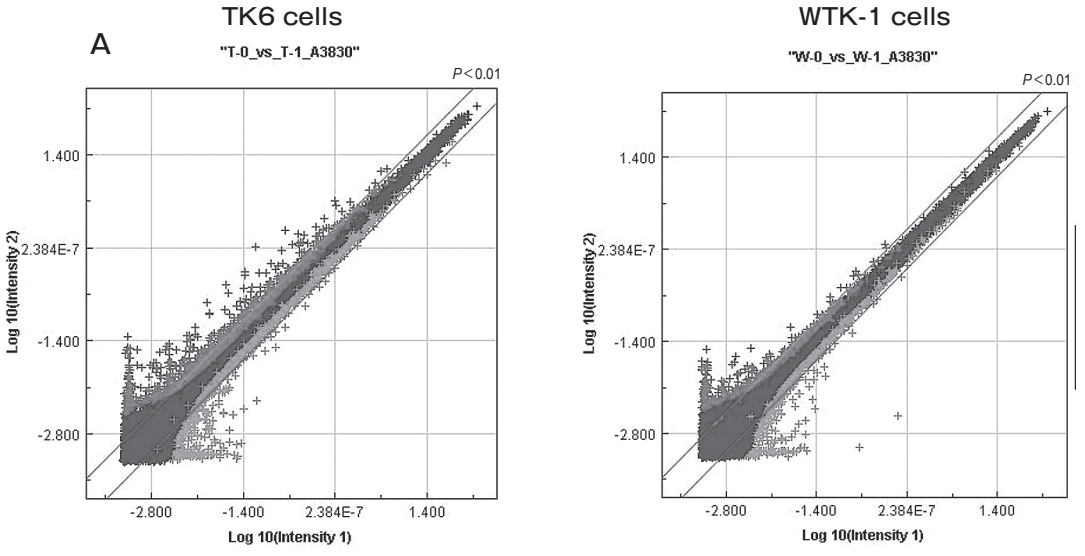
Fig. 2 *In vitro* micronucleus assay in TK6 and WTK-1 cells after 5-FU treatment for 24 h. MN+ cells, The frequency of the cells having micronucleus 5-FU increased the MN frequency in WTK-1 cells, to 2.7, and 4 times the control value at 0.5 and 1.0 μg/mL of 5-FU, respectively. In TK6 cells, 5-FU increased MN frequency (2.0 times of the control value) at 1.0 μg/mL only.
*significant difference ($p < 0.05$) from the vehicle control by the conditional binomial test.

rate was decreased significantly in 1.0 and 2.0 μg/mL in WTK-1 cells, while that in TK6 cells was decreased significantly in 0.125 μg/mL or more ($p < 0.05$). Thus, the cytotoxicity induced by 5-FU was more severe in TK6 cells than in WTK-1 cells. In WTK-1 cells, 5-FU increased the mutation frequency significantly compared with the solvent control at concentrations of 1.0 μg/mL or more and was judged as positive in *Tk* mutation assay ($p < 0.05$) (Fig. 1C). However, 5-FU was not mutagenic to TK6 cells.

5-FU increased the frequency of cells with micronucleus (MN frequency) in WTK-1 cells in a concentration-dependent manner, increasing the MN frequency to 2.7 and 4.0 times the control value at 0.5 and 1.0 μg/mL, respectively ($p < 0.05$) (Fig. 2). In TK6 cells, 5-FU increased the MN frequency (to 2.0 times the control value) at 1.0 μg/mL only.

Microarray analysis. The changes of gene expression were analyzed in a 5-FU treatment group compared with the solvent control. The numbers of up-regulated (more than 2-fold and *p*-value < 0.01) genes were 520 and 245 in TK6 and WTK-1 cells, respectively, and the numbers of down-regulated (less than 0.5-fold and *p*-value < 0.01) genes were 271 and 212 in TK6 and WTK-1 cells, respectively (Fig. 3). The possible function of the up-regulated genes in TK6 cells treated with 5-FU is shown in Table 1. In TK6 cells, the genes related to the *TP53* signaling pathway, involved in processes such as apoptosis and DNA damage response, were up-regulated, but this was not the case in WTK-1 cells. Namely, the *BAX*, *BBC3*, *FAS*, *SESN1*, *SESN2*, *p21* (*CDK1A*), *DDB2*, and *GADD45A* genes were up-regulated after 5-FU treatment in TK6 cells (Table 2 and Fig. 4). The genes involved in the negative feedback to the *TP53* pathway (*MDM2* and *Cyclin G*) were also up-regulated after 5-FU treatment in TK6 cells. In WTK-1 cells, however, the genes related to the *TP53* pathway or negative feedback to the *TP53* pathway were not changed. None of the genes related to cytotoxicity, cell death, or gene mutation were found in down-regulated genes in TK6 cells or WTK-1 cells (data not shown).

Quantitative RT-PCR. The *BAX*, *p21*, and *MDM2* genes were selected as target genes for analysis of quantitative RT-PCR because all are key genes for apoptosis, cell cycle regulation, and negative feedback to the *TP53* gene, and all showed marked elevation in gene expression after 5-FU treatment in the microarray analysis. The expressions of the *BAX*, *p21*, and *MDM2* genes after 5-FU treatment are shown in Fig. 5. The expression increased significantly in



B

TK6	Gene
Up	520
Down	271

WTK-1	Gene
Up	245
Down	212

Fig. 3 The changes of gene expression in the cells treated with 5-FU for 24 h. The changes of gene expression between 5-FU treatment group with the solvent control group (**A**), the numbers of genes changed expression more than 2-fold and *p*-value 0.01 compared with control (**B**). The numbers of up-regulated (more than 2-fold and *p*-value < 0.01) genes were 520 and 245 in TK6 and WTK-1 cells, respectively, and the numbers of down-regulated (less than 0.5-fold and *p*-value < 0.01) genes were 271 and 212 in TK6 and WTK-1 cells, respectively.

Table 1 Changes in mRNA expression related to *TP53* activity in TK6 cells after 5-FU treatment (Up-regulation)

Function	Gene counts	*p*-value
TP53 signaling pathway	15	<0.00001
Regulation of cell proliferation	34	<0.00001
Induction of apoptosis by intracellular signals	8	0.00004
DNA damage response, signal transduction by TP53 class mediator	6	0.00007
Regulation of apoptosis	30	0.00013
Response to DNA damage stimulus	17	0.00074

In TK6 cells, the genes implicated in the *TP53* signaling pathway, apoptosis, and DNA damage response were changed in 5-FU-treated group compared with the solvent control group but not in WTK-1 cells. The analysis was performed by the DAVID bioinformatics resources ver. 6.7.

TK6 cells ($p < 0.05$) but not in WTK-1 cells. Thus, these results confirmed the representative results of the microarray analyses.

Discussion

In this study, the relationship between cytotoxicity or mutagenicity and genetic status of the *TP53* gene was analyzed in 2 cell lines with or without *TP53* mutation after 5-FU treatment. Both TK6 and WTK-1 cells are derived from WIL-2 cells [5], but only WTK-1 cells have the homozygous mutant *TP53* gene [6]. A fluorinated pyrimidine base analogue, 5-FU, is converted to 5-fluoro-2-deoxyuridylate monophos-

Table 2 Up-regulated mRNA involved in the *TP53* pathway, apoptosis and cell cycle in TK6 cells

Gene Name	Gene Symbol	TK6 cells	WTK-1 cells
		Fold Change	Fold Change
BCL2 binding component 3	BBC3	3.83	−1.06
BCL2-associated X protein	BAX	2.36	−1.16
Cyclin G1	CCNG1	2.40	−1.06
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	5.73	1.48
Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	CDKN2B	11.78	1.08
Damage-specific DNA binding protein 2, 48kDa	DDB2	2.76	−1.06
Fas (TNF receptor superfamily, member 6)	FAS	2.28	1.06
Forkhead box O1	FOXO1	2.18	1.04
Growth arrest and DNA-damage-inducible, alpha	GADD45A	2.79	1.09
Leucine-rich repeats and death domain containing	LRDD	2.78	−1.01
MAX dimerization protein 4	MXD4	2.29	−1.16
Mdm2 TP53 binding protein homolog (mouse)	MDM2	3.82	1.15
Homo sapiens hypothetical protein MGC5370, mRNA (cDNA clone IMAGE: 3049213), partial cds. [BC006795]	MGC5370	4.81	1.26
Nuclear protein, transcriptional regulator, 1	NUPR1	12.84	1.97
Pleckstrin homology-like domain, family A, member 3	PHLDA3	3.79	1.48
Polymerase (DNA directed), eta	POLH	2.46	−1.35
Protein phosphatase 1D magnesium-dependent, delta isoform	PPM1D	2.02	1.10
Sestrin 1	SESN1	6.14	−1.04
Sestrin 2	SESN2	4.41	−1.05
TP53 regulated inhibitor of apoptosis 1	TRIAP1	2.41	−1.02
Tumor necrosis factor (ligand) superfamily, member 4	TNFSF4	11.38	1.66
Tumor necrosis factor receptor superfamily, member 4 mRNA for OX40 homologue. [X75962]	TNFSF4 X75962	5.34	1.30
Tumor protein TP53 inducible nuclear protein 1	TP53INP1	8.22	−2.14
Tumor protein TP53 inducible protein 3	TP53I3	16.39	1.62
Xeroderma pigmentosum, complementation group C	XPC	2.48	−1.15
Zinc finger, matrin type 3	ZMAT3	4.54	1.13

The up-regulated (more than 2-fold and p-value < 0.01) genes involved in the *TP53* pathway after 5-FU treatment compared with the solvent control were shown. The genes up-regulated in TK6 cells were hardly up-regulated in WTK-1 cells. (Analysis by the DAVID bioinformatics resources ver. 6.7.)

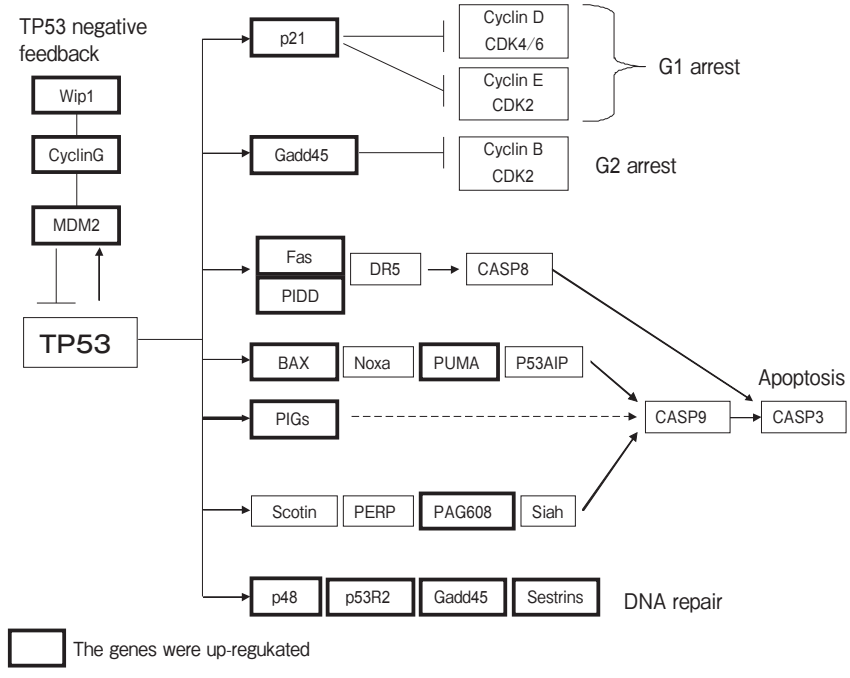


Fig. 4 Change of gene expression in the *TP53* pathway in TK6 cells after 5-FU treatment for 24 h.

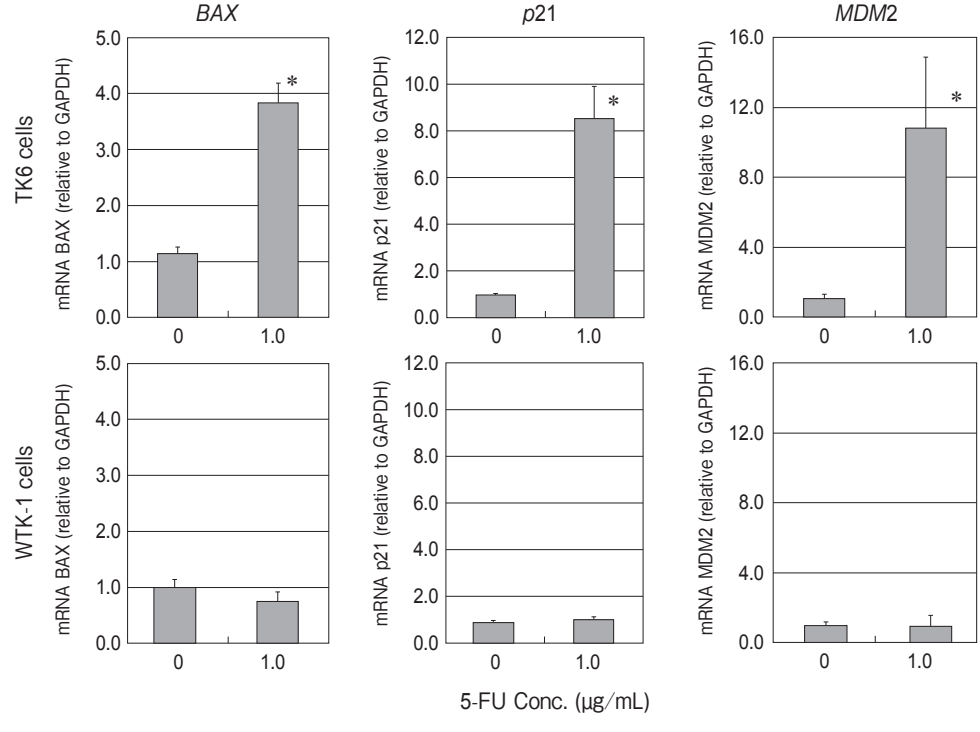


Fig. 5 The expression of mRNA after 24 h 5-FU treatment on TK6 and WTK-1 cells. The expressions of mRNA are shown in TK6 cells (upper column) and WTK-1 cells (lower column). The expressions of *BAX*, *p*21, and *MDM*2 genes were up-regulated with greater frequency in TK6 cells than in WTK-1 cells.
*significant difference ($p < 0.05$) from the vehicle control by Student's *t*-test.

phate (FdUMP). FdUMP complexes with TS and 5, 10-methylene tetrahydrofolate (CH_2FH_4) and competitively inhibits DNA synthesis [19–21, 29]. Thymidine depletion by TS inhibition induces a nucleotide pool imbalance and the misincorporation of dUTP and/or misrepair of the uracil-containing lesion, causing double-strand DNA breaks (DSBs) [30–32]. The direct incorporation of 5-fluoro-deoxyuridine triphosphate (FdUTP) inhibits DNA synthesis [20]. Therefore, the intracellular metabolism of 5-FU to FdUMP or FdUTP leads to cytotoxic and mutagenic effects. 5-FU is phosphorylated by orotate phosphoribosyl transferase (OPRT) to fluoro-uridylate monophosphate and detoxified by dihydrouracil dehydrogenase (DPD) to fluoro-β-alanine. TS, OPRT, and/or DPD are the predictive factors for the response to 5-FU and its derivative drugs [33, 34]. Compared with WTK-1 cells, TK6 cells had much lower DPD activity, and 5-FU in TK6 cells was efficiently metabolized to FdUMP without being detoxified. The TS content was higher in TK6 cells than in WTK-1 cells, so 5-FU metabolism would not be very different in TK6 and WTK-1 cells [25].

In general, X-rays and mutagens induce more cytotoxicity and/or a higher frequency of apoptosis in cells with wild-type *TP53* than in *TP53* mutant cells, and a higher frequency of mutations and chromosomal aberrations in *TP53* mutant cells than in wild-type cells [14]. In this study, the cytotoxicity induced by 5-FU was also more prominent in TK6 cells (*TP53* wild type) cells than in WTK-1 cells (*TP53* mutant). Mutation frequency and chromosomal damage (MN frequency) occurred more in WTK-1 cells than in TK6 cells after 5-FU treatment. These results were similar to the response to X-ray or mutagens [8, 9].

In microarray analysis of gene expression, a larger number of genes were up-regulated in TK6 cells than in WTK-1 cells; however, the numbers of down-regulated genes were almost equal. *BAX*, *BBC3*, *FAS*, *SESN1*, *SESN2*, *p21*, *DDB2*, and *GADD45A* were up-regulated after 5-FU treatment in TK6 cells but not in WTK-1 cells. These genes were implicated in the *TP53* pathway (apoptosis, cell cycle, or DNA repair). *MDM2* and *Cyclin G*, involved in the negative feedback to the *TP53* pathway, were also up-regulated after 5-FU treatment in TK6 cells. In quantitative RT-PCR analyses, the up-regulation of *BAX*, *p21*, and *MDM2* were ensured in TK6 cells but not in WTK-1 cells after 5-FU treatment. Zschenker *et al.* reported that an especially strong induction was seen for the *p21*, *MDM2*, *SESN1*, *XPC*, *DDB2*, and *FUCA1* genes in TK6 cells after X-ray irradiation [9]. The direct DNA damaging agents also increased the expression of genes associated with the *TP53* pathway related to regulation of the cell cycle or apoptosis in TK6 cells [8]. These results are consistent with our results.

In our previous study, 5-FU induced the apoptotic cells in TK6 cells rather than WTK-1 cells after 5-FU treatment [25]. Inhibition of DNA synthesis by 5-FU leads to nucleotide pool imbalance and induces DSBs. When DNA was injured, ATM phosphorylates TP53 protein, and phosphorylated TP53 protein induces BAX, FAS, and so on, which leads to the induction of apoptosis [35–37]. The increase of apoptosis in TK6 cells in previous studies might have been due to the up-regulation of genes involved in the *TP53* pathway.

After 5-FU treatment, G_1 phase cells were accumulated in TK6 cells, whereas S phase cells were accumulated in WTK-1 cells [25]. Phosphorylated TP53 protein up-regulates the *p21* gene, which inhibits the effect of CDK4 and leads to the G_1 phase arrest [38, 39]. The differences in cell cycle arrest in TK6 and WTK-1 cells might be related to the differences in gene expression between these cell lines.

No evidence that 5-FU is carcinogenic has been found in mice or rats. In clinical trials, there is no epidemiological study of 5-FU as a single agent. However, 5-fluoro-2'-deoxyuridine, which has the same pharmacodynamic action as 5-FU, was reported not to increase the risk of second malignancies. Therefore, 5-FU has been listed as not classifiable regarding its carcinogenicity to humans by the International Agency for Research on Cancer (IARC) [40]. This classification appears to conform with our results that 5-FU induced no gene mutation and up-regulated many genes involved in the *TP53* pathway in TK6 cells (*TP53* wild type). However, in WTK-1 cells (*TP53* mutated cells), gene mutation was induced by 5-FU, and a few genes related to the *TP53* pathway were expressed. The cells with mutated *TP53* or defective DNA repair might show gene mutation (perhaps leading to carcinogenicity and/or cancer progression) by 5-FU treatment.

The mutation frequency in *Tk* mutation assay is known to be induced by point mutation or chromo-

somal aberration [41]. In this study, it was unclear whether 5-FU induced point mutation or chromosomal aberration in mutated cells, because the sequence in mutated genes was not analyzed. However, 5-FU did not induce point mutation in *Salmonella typhimurium* [22] and increased the MN frequency, which indicates the chromosomal aberration in WTK-1 cells but not in TK6 cells. Therefore, mutation frequency increased by 5-FU was caused due to the chromosomal aberration, and the difference in degree of MN frequency between TK6 cells with WTK-1 cells might affect mutation frequency.

In the present study, 5-FU induced gene mutation and chromosomal damage in *TP53* mutated WTK-1 cells but not *TP53* wild-type TK6 cells after 24 h treatment. These differences in response to 5-FU were suggested as being due to the difference in gene expression related to the *TP53* pathway, especially the induction of apoptosis or cell cycle arrest after the DNA damage. Since our results demonstrated that 5-FU exerts less effective cytotoxicity and stronger mutagenic effects on *TP53*-mutated cells than on *TP53*-wild type cells, the clinical efficacy of 5-FU will likely be higher in *TP53*-wild-type cancers than in *TP53*-mutated cancers and the *TP53* gene status may be responsible for the response to 5-FU therapy and the patient prognosis. This important prediction has been recently confirmed on patients with squamous cell carcinoma of the esophagus [42].

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