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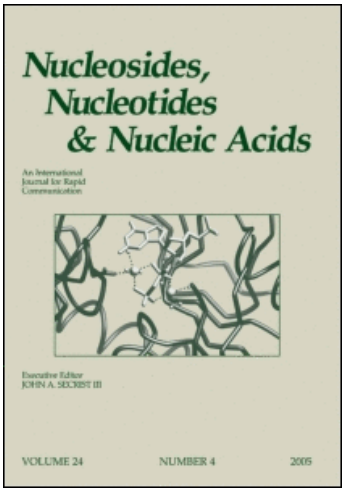

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Sequence Analysis of the 5'-Flanking Regions of Human Dihydropyrimidine Dehydrogenase Gene: Identification of a New Polymorphism Related with Effects of 5-Fluorouracil

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SEQUENCE ANALYSIS OF THE 5′-FLANKING REGIONS OF HUMAN DIHYDROPYRIMIDINE DEHYDROGENASE GENE: IDENTIFICATION OF A NEW POLYMORPHISM RELATED WITH EFFECTS OF 5-FLUOROURACIL

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▫ *Dihydropyrimidine dehydrogenase (DPD), known as a rate-limiting metabolic enzyme in the catabolism of 5-fluorouracil (5-FU), degrades more than about 80% of the administered 5-FU in human liver. Since it was reported that the anticancer effects of 5-FU were observed in cancer patients with lower DPD activities, many attempts have been conducted to anticipate the expected anticancer effects of 5-FU based on expression of intracanceral DPD. It have been reported that 39 different mutations and polymorphisms in the coding regions of DPD genes have been identified; however, there is no report on polymorphisms in the 5′-flanking region of DPD genes. We investigated polymorphisms in the 5′-flanking regions (3,058 bp), which are considered to control expression of DPD genes, in genomic DNA extracted from 37 kinds of human cancer cells. As the results, out of 37 cancer cells subjected to analysis, DLD-1 cells had C insertion and 7 strains G deletion, which were hetelozygote. No significant relationship was identified between the DPD activity and the expression levels of DPD mRNA in examined 10 kinds of human cancer cells. However, in DLD-1 cells, which have C-insertion polymorphism in 5′-flanking region of DPD gene, the DPD activity was below detection limit ($\leq$ 0.5 pmol/min/mg protein). Furthermore, 50% of cytosine residue on the CpG site generated by the C insertion was methylated at the 5 position. In this study, we have identified novel polymorphism possibly related the cytotoxicity of 5-FU in the 5′-flanking region of DPD gene. It is suggested that newly identified polymorphism of DPD gene might affect transcription of DPD, thereby providing influence on the clinical outcome of cancer patients treated with 5-FU.*

Keywords Dihydropyrimidine Dehydrogenase, 5-Fluorouracil, Polymorphism

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INTRODUCTION

5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic drugs to treat cancer patients. Two explanations have been given for the cytotoxic action of 5-FU: one widely accepted explanation is that 5-fluorodeoxyuidine 5′-monophosphate (FdUMP), an active metabolite of 5-FU, inhibits thymidylate synthase (TS) irreversibly. Another explanation is that 5-FU is incorporated into RNA and distorts gene expression.

However, more than 80% of 5-FU administered is rapidly degraded to an inactive form by dihydropyrimidine dehydrogenase (DPD), mainly in the liver. Regarding to expression of DPD, recent studies have revealed that a deficiency of liver DPD activity induce the lethal toxicity in cancer patients following administration of consecutive 5-FU,[1] and that DPD in human cancer cells is one of response-limiting factors of 5-FU. Therefore, measuring the expression of DPD at gene and/or protein levels is important to predict the possible toxicity or sensitivity of 5-FU in cancer patients.

Human DPD gene is present as a single copy gene on chromosome1p22 and consists of 23 exons.[2,3] It has been reported that 39 different mutations and polymorphisms have been identified in DPD gene.[4–10] IVS14 + 1G > A mutation is the most commonly observed and it has been reported that the mutation is often found in the patients suffering from severe toxicity after 5-FU administration.[11–13] IVS14 + 1G > A mutation induces splicing abnormality, leading to loss of the segment (exon 14) consisting of 165 bases encoding amino acids. As a result, the DPD mRNA lacks the function of DPD.[14] In contrast, there is no report about polymorphism in the promoter regions.

In this study, we report the sequence analysis of the 5′-flanking regions comprising 3,058 bp that are considered to control regulation of DPD gene, with use of the genomic DNA that was extracted from 37 kinds of the human cancer cells. We also report a novel polymorphism in 5′-flanking region of DPD gene related with 5-FU activity.

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METHODS

Cancer Cell Lines

The human lung cancer cell line A549, the human colon cancer cell line DLD-1, the human breast cancer cell line MCF-7, the human pancreas cancer cell line BxPC-3 and MIAPaCa-2 were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan); the human stomach cancer cell line TMK-1 was obtained from National Cancer Center (Tokyo, Japan); the human colon cancer cell line KM12C was obtained from National Cancer Center Research Institute (Tokyo, Japan); the other 30 kinds of human cell lines were supplied by the Japanese Cancer Research Resources Bank (JCRB).

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PCR and Sequencing Analysis

DNA from cultured human cancer cells (2×10^6 cells) was prepared using DNeasy Tissue Kit (QIAGEN). PCR reactions were carried out in 50 μL volumes comprising 0.1 μg of DNA preparation, 0.1 U of AmpliTaq Gold (Applied Biosystems), primers at a final concentration of 0.5 pmol/μL, Mg^{2+} at a final concentration of 1.5 mM, 1 × PCR buffer, dNTP at a final concentration of 0.2 mM each. A total of 30 cycles were carried out (94°C for 1 min, 56–58°C depending on primer pairs for 1 min, and 72°C for 2 min, final extension for 7 min) following hot-start at 94°C for 7 min. To purify PCR products and to remove abnormal DNA fragments, QIA quick Gel Extraction Kit (QIAGEN) was used. After purification, cycle sequencing reactions were performed using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Amplification products were analyzed by capillary electrophoresis (ABI PRISM® 310 Genetic Analyzer, Applied Biosystems).

Sensitivity for 5-Fluorouracil

Cancer cells (2,000 cells/well) in the exponential growth phase were cultivated in 96-well plates. Twenty-four hours after plating, the cells were exposed to 5-FU for 4 days. The cytotoxic effect of 5-FU was measured by dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay. IC_{50} values were estimated from the regression line of log-logit plots of concentration versus growth inhibition rate.

Bisulfite Treatment and Methylation Analysis

Genomic DNA was reacted with bisulfite using the EZ DNA Methylation Kit™ (ZYMO RESEACH). PCR were performed on bisulfite-reacted DNA. Primer sequences were designed in the 5′-flanking regions containing generated CpG site.

(F) 5′-GTTAAGTGTATGAAGGAGTGTCG

(R) 5′-AATAAAAAATACCAAAACACCGAT

PCR amplification of the selected region (−896 to −453) in the 5′-flanking regions of *DPD* gene yield 444 bp product with primer sets was performed under the following conditions: a total of 35 cycles were carried out (94°C for 1 min, 48°C for 1 min, and 72°C for 2 min, final extension for 7 min) following hot-start at 94°C for 7 min. After purification of the PCR product, cycle sequencing reactions were conducted using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Amplification products were analyzed by capillary electrophoresis (ABI PRISM® 310 Genetic Analyzer, Applied Biosystems).

Measurement of DPD Activity

The enzyme assay, a modification of the method introduced by Naguib and colleagues,[15] has been previously described in detail in Takechi et al.[16] Briefly,

cancer cells were harvested by trypsinization before reaching confluence (approximately 70% confluence), freeze-thawed in two volumes of homogenization buffer, and centrifuged at 105,000 × *g* for 1 h at 4°C. The supernatant fluid (cytosol) was collected as the enzyme source. The enzyme reaction mixture, which contained 10 mM potassium phosphate (pH 8.0), 0.5 mM ethyldiamine tetra acetic acid (EDTA), 0.5 mM 2-mercaptoethanol, 2 mM dithiothreitol, 5 mM $MgCl_2$ 20 μM [6-^{14}C]5-FU (56 mCi/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO), 100 μM NADPH (Sigma Chemical Co., St. Louis, MO), and 25 μL of cytosol in a final volume of 50 μL, was incubated at 37°C for 30 min. DPD activity was determined by measuring the sum of the products formed from 5-FU, i.e., dihydrofluorouracil, α-fluoro-β-ureidpropionate, and α-fluoro-β-alanine. After chemical hydrolyzation and neutralization using KOH and $HClO_4$, a 5 μL aliquot of the supernatant was applied to a thin-layer chromatography plate (silica gel 60 F254, Merck, Darmstadt, Germany) and developed with a mixture of ethanol and 1 M ammonium acetate (5:1,v/v), according to the method of Ikenaka and colleagues.[17] Each product was visualized and quantified using an imaging analyzer BAS-2000 (Fujix, Tokyo, Japan).

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Customized DNA Array

Target DNA made from the DPD gene was immobilized on a glass plate. Target DNA was designed based on sequence homology analysis to minimize cross-hybridization with other genes and was practically tested by Northern blot. The basic technology of the customized DNA array is almost the same as that of a Stanford-type cDNA microarray (Dr. Brown's Lab protocol, http://cmgm.stanford.edu/pbrown/protocols/index.htm). Samples of the cell lines were stored at −80°C until use. Frozen tissues were suspended in Buffer RLT (Qiagen, Hilden, Germany) and homogenized using a Mixer Mill MM300 (F. Kurt Retsch GmbH & Co., Haan Germany). RNA extraction was performed using an RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA quality was judged from the relative intensities of the 28S and 18S ribosomal RNA bands after agarose gel electrophoresis. Purified total RNA (20 μg) was incubated at 70°C for 5 min and cooled on ice. It was reverse-transcribed with a mixture of specific primers and 200 units of PowerScript reverse transcriptase, and incubated 42°C for 1.5 h. The cDNA was labeled using Cy5 (Cy5 monofunctional reactive dye, Amersham, Cat No. PA25001), and purified by a Nucleo Spin Extract kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany). Labeled cDNA was hybridized in 6X SSC, with 0.2% SDS, 0.01 mg/mL Human Cot-l DNA and 5X Denhalt's solution for 16 h at 60°C for spotted cDNA arrays. The slides were washed in 2X SSC with 0.2% SDS at 60°C twice, and finally 0.05X SSC at room temperature and scanned using a Scanner FLA-8000 (FujiFilm, Tokyo, Japan). Data were analyzed using Array Gauge (FujiFilm, Tokyo, Japan).

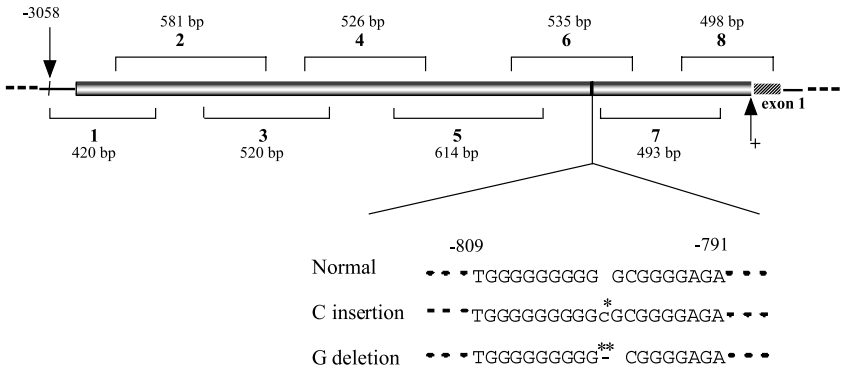

FIGURE 1 The scheme of divided 5′-flanking region of *DPD* gene into eight regions. The upstream region of *DPD* gene was divided into eight regions. Each region was amplified by PCR. *: C-insertion. **: G-deletion.

Statistical Analysis

Regression analysis was performed mRNA expression of a cancer cell line using Microsoft Excel 2002 software (Microsoft Co.).

RESULTS

Using the genomic DNA extracted from 37 kinds of human cancer cells, we performed sequence analysis on the 5′-flanking regions of the *DPD* gene. Based on the sequence of the human chromosome 1 registered in NCBI (National Center for

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TABLE 1 Sequences of PCR Primers for 5′-Flanking Region of *DPD* Gene

Region no.	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplified region
1	TAAGCTGTTTCTCCAAAGCCTAC	AGAGGATAAGTCCAATATACGGTG	− 3058 to − 2639
2	CACGTGAGCAATGTGATTAGATG	CTAGATCCTCTTGGAGATATAGAG	− 2808 to − 2228
3	CCTGAAAGAGCAATAAGCCTGTAT	CACTTGGTGATTGAATGAACAGAG	− 2505 to − 1986
4	GTGACCTATTTCTCAGCAGTAAGT	TAACCGCTGAATAACCTGCTCTA	− 2106 to − 1581
5	AAGTGGAGGGCAATGTGCAAA	AAATGTGATGCCTGCTTGTAAGATA	− 1707 to − 1094
6	CAGGTTCTGGAAGGTAATCT	TTGCTCTAGAGCTTGCTGAG	− 1212 to − 678
7	GAGAGGTCTGGAGTACACATAT	CTCCAAGCAACAACTTGCACTTT	− 784 to − 292
8	CATCCTTGAGGAGTTCCTGAA	CATGGCAGTGCCTACAGTCT	− 396 to + 102

TABLE 2 Result of Sequencing Analysis for 5′-Flanking Region of *DPD* Gene

Cell line	Origin	Region no. −809 to −798
A549	Lung	G-deletion/normal
Lu-99		Normal
NCI-H526		Normal
SBC-1		Normal
EBC-1		Normal
4-1ST	Stomach	G-deletion/normal
AZ-521		G-deletion/normal
MKN28		Normal
MKN45		Normal
TMK-1		G-deletion/normal
DLD-1	Colon	C-insertion/normal
HCT-116		Normal
KM12C		Normal
LoVo		Normal
WiDr		Normal
T-47D	Breast	Normal
MDA-MB-231		Normal
MDA-MB-435		Normal
MCF-7		Normal
HeLa S3	Uterus	Normal
HeLa		Normal
SKOV-3	Ovary	Normal
PC-3(P)	Prostate	Normal
DU145		Normal
TSU-Pr1		G-deletion/normal
LNCaP		Normal
KB	Head and neck	Normal
JHH-7	Hepatoma	Normal
JHH-5		Normal
BxPC-3	Pancreas	Normal
CFPAC-1		Normal
PANC-1		Normal
MIAPaCa-2		G-deletion/normal
KP-2		Normal
AsPC-1		Normal
A431	Skin	Normal
HT1080	Fibrosarcoma	G-deletion/normal

The results of sequence analysis, G-deletion were detected in 6 kinds of cells on −809 to −798 region, and C-insertion was detected in DLD-1 cells between −800 and −799. All of polymorphisms detected were heterozygote.

Biotechnology Information), 3058 bp upstream from 5′ end of the exon 1 of the *DPD* gene (Accession No. NM_000110) was divided into 8 fragments, and then the primer was prepared to permit overlapping of each region by approximately 100 bp (Figure 1, Table 1). As the result of the sequence analysis of respective regions on both from forward and reverse directions, we detected two new polymorphisms that have not been reported. These new polymorphisms were included C-insertion between −800 and −799 in DLD-1 cells and G-deletion at the locations of −809 to

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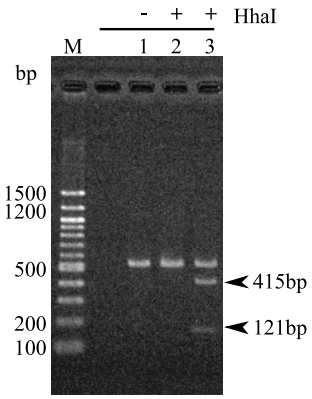

FIGURE 2 PCR-RFLP analysis of a polymorphisms detected −809 to −791 in the human DPD 5′-flanking region. Lane M: 100 bp DNA ladder marker. Lane 1: PCR product of DLD-1 cells (no digestion with Hha I), Lane 2: PCR product of A549 cells (digestion with Hha I), Lane 3: PCR product of DLD-1 cells (digestion with Hha I). Each PCR products of the region 6 was digested at 37°C for 1 hr with 10 U of Hha I (TaKaRa Bio Inc.). After stop enzyme reaction, the sample was evaluated by 1.6% agarose gel electrophoresis.

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−798 in 7 kinds of cells (A549, 4-1ST, AZ-521, TMK-1, TSU-Pr1, MIAPaCa-2, HT1080) as shown in Figure 1, which were heterozygote (Table 2). The sequence of other 5′-flanking regions of the *DPD* gene was identical with NCBI database.

The cutting site of region 6 by restriction enzyme Hha I (–CGCG–) was generated by the C-insertion. After the digestion, two fragments (415 and 121 bp) and the 536 bp fragment in lane 3 of Figure 2 were observed. We carried out sequence analysis for the 536 bp DNA fragment of lane 3 that was excised from the agarose gel to eliminate the possibility that the 536 bp fragment of lane 3 remained by defective digestion. The results of sequencing for the fragment did not represent

TABLE 3 Correlation of DPD Activity and DPD and TS mRNA Levels and Polymorphisms

Cell line	Origin	DPD activity (pmol/min/mg protein)	DPD mRNA (DPD/ GAPDH)	TS mRNA (DPD/ GAPDH)	IC_{50} of 5-FU (μM)	Genotype
A549	Lung	30.93	0.62	0.50	1.70	G-deletion/normal
Lu-99		78.46	0.93	1.22	5.49	Normal
DLD-1	Colon	$\leq 0.5^{*}$	0.69	2.10	7.80	C-insertion/normal
KM12C		2.36	1.71	0.90	6.20	Normal
PC-3(P)	Prostate	30	1.52	3.01	131	Normal
DU145		3.76	0.94	1.82	20	Normal
TSU-Pr1		41.85	0.78	2.09	17.50	G-deletion/normal
KB	Head and neck	31.16	1.04	1.74	26.90	Normal
BxPC-3	Pancreas	225.99	1.87	1.31	138	Normal
MIAPaCa-2		197.45	1.17	1.74	22	G-deletion/normal

Each expression level of DPD and TS was standardized with GAPDH mRNA quantity as the endogenous control.

*Below detection limit.

the C-insertion, from which the polymorphism of DLD-1 cells was heterozygote (C-insertion/normal). By C-insertion in DLD-1 cells, a new CpG site was generated. The sequence analysis of the bisulfite-treated genomic DNA of DLD-1 cells revealed that 50% of the cytosine residue in the CpG site was methylated. The deamination by bisulfite treatment occurred on the other sites at 100% (data not shown). Table 3 shows the expression levels of TS and DPD mRNAs, DPD activity, and the cytotoxicity of 5-FU expressed as IC_{50} values. No significant relationship was seen between the expression levels of DPD mRNA and the activity of DPD in 10 human cancer cells used. Therefore, it seemed to be impossible to estimate the expression level of DPD proteins from its gene expression level. In only DLD-1 cancer cells, low expression of DPD mRNA (relative value of 0.69) led to under-detectable activity of DPD (< 0.5 pmol/min/mg protein). Although the cytotoxicity of 5-FU is possibly depend on the expression of not only DPD but also TS and other 5-FU-metabolizing enzymes, IC_{50} value of 5-FU in DLD-1 cells was tended to be low compared with other cancer cells such as TSU-Pr1, KB, BxPC-3, and MIAPaCa-2 with higher activity of DPD. There were no correlation between expression levels of mRNA and the activity in cancer cells with G-deletion in 5′-flanking region of DPD gene.

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DISCUSSION

DPD is the initial rate-limiting enzyme in the degradation of 5-FU and the enzyme inactivates more than 80% of the administrated dose of 5-FU. DPD is also known to be a principal factor of 5-FU pharmacokinetics, clinical toxicity, and drug resistance. The activity of DPD can be detected in various tissues[15,18] and the activity of DPD in normal liver is well correlated with that of peripheral blood mononuclear cells, whereby the latter activity is used in place of the DPD activity of the whole body.[19] The DPD activity demonstrates considerable variation in healthy and cancer populations.[20] It has been reported that patients have reduced DPD activity, which is associated with severe 5-FU toxicity in cancer patients.[21] Information regarding TS cancer tissue levels is also important because low levels of cancer TS expression are observed in patients receiving 5-FU-based chemotherapy.[22,23] The balance between DPD and TS is related to the overall outcomes of anticancer effects in chemotherapy with 5-FU.

In this study, we detected novel polymorphisms as shown in Figure 1. As the results, out of 37 human cancer cells subjected to analysis, 1 strain (DLD-1 cells) had C-insertion and 7 strains had G-deletion. Results of sequencing analysis and RFLP analysis disclosed that all polymorphisms detected were heterozygote (Table 2). No significant relationship was observed between the DPD activity and the expression levels of DPD mRNA in examined 10 human cancer cells (Table 3). However, in DLD-1 cells, which have C-insertion polymorphism, the DPD activity was below detection limit ($\leq$0.5 pmol/min/mg protein). Furthermore, methylation analysis on the CpG site generated by the C-insertion revealed occurrence of 50% of methylation of the cytosine residue. McLeod et al. and Takechi et al. suggested the

possibility of downregulation of DPD in cancer cells.[24–26] Since the DPD activity and the protein content were independent of mRNA levels (Table 3), it appears that DPD protein formation may be controlled at the posttranscriptional levels. Methylated DNA that was reported to prevent the gene expression was observed in the 5′-flanking regions of gene.[27] If this C-insertion is homozygote, it might provide some correlation between the expression and the activity of DPD. We have supposed that the C-insertion might be participated in a control at the posttranscriptional levels of DPD protein biosynthesis, thereby providing influence on the clinical outcome of cancer patients treated with 5-FU. The C-insertion occurrence and following methylation of 5-position of cytosine residue might be associated with loss of the sequence of binding site for transcription factors. It may be conceivable that prevention of binding a transcription factor with methylated newly generated CpG site that probably affected the DPD gene regulation. In all cells investigated in the study, there were no IVS14 + 1G > A polymorphisms (data not shown).

In this study, we demonstrated a new polymorphism observed in the 5′-flanking regions of DPD gene and provided a possibility that the polymorphism in the promoter regions conducive to control of the DPD gene regulation gives useful findings for the chemotherapy with 5-FU. It is necessary to advance further research in order to clarify the relation between the polymorphism and DPD expression mechanisms.

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