SPANDIDOS PUBLICATIONS

The relationship between 5-fluorouracil sensitivity and single nucleotide polymorphisms of the orotate phosphoribosyl transferase gene in colorectal cancer

MASAYUKI KITAJIMA[1], NAOHITO TAKITA[1], MASAKI HATA[1], TSUTOMU MAEDA[1], KAZUHIRO SAKAMOTO[1], TOSHIKI KAMANO[1] and TAKUMI OCHIAI[2]

[1]Department of Surgery, Juntendo University School of Medicine; [2]Department of Surgery, Tobu Chiik Hospital, Tokyo Metropolitan Health and Medical Treatment Corporation, Tokyo, Japan

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Abstract. Orotate phosphoribosyl transferase (OPRT) is an enzyme playing an important role in exertion of the effect of 5-fluorouracil (5-FU). A type of gene polymorphism, single nucleotide polymorphism (SNP), is considered to be a factor affecting individual differences in exertion of drug effects, and its analysis has recently made progress. We investigated the correlation between SNP of OPRT and 5-FU sensitivity in colon and rectal cancers. The subjects were 31 patients with colorectal cancer who underwent surgical excision between December 2003 and July 2004 at our department. Of SNP of OPRT, 638G/C, 1050T/A, and 1336A/G located in the coding region were analyzed by invader assay. The growth inhibition rate (% IR) of colorectal cancer by 5-FU was obtained by the CDDST method, and 5-FU sensitivity was compared among strains (wild-, homo-, and hetero-types) of each polymorphism. There was no relationship between the strains and 5-FU sensitivity in any of the SNPs. The investigated SNPs of OPRT may have no major influence on 5-FU sensitivity. However, there are many unknown factors in the relationship between SNP of OPRT and 5-FU sensitivity, and SNP analysis of other regions is necessary.

Introduction

5-FU is phosphorylated by various enzymes in cells to produce activated forms (FUTP and FdUMP) and exhibits an antitumor effect. After incorporation into cells, 5-FU is phosphorylated mainly by 3 pathways. Among the 3 pathways, the pathway by OPRT is considered most important for exertion of the 5-FU effect (1; Ochiai *et al*, Proc ASCO: abs. 3574, 2004).

Correspondence to: Dr Toshiki Kamano, Department of Surgery, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyou-ku, Tokyo 113-8421, Japan
E-mail: kapira@med.juntendo.ac.jp

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The effects of many drugs, including anticancer drugs, vary among individuals in routine clinical practice. Various gene polymorphisms are considered to be involved in this variation; SNP has been analyzed and is attracting the most attention (2). We investigated the relationship between SNP located in the coding region (638G/C, 1050T/A, and 1336A/G) that may affect the exertion of drug effects and 5-FU sensitivity in colon and rectal cancers.

Patients and methods

Patients. Surgical specimens were obtained from 31 patients with colorectal cancer who underwent surgical excision between December 2003 and July 2004 at our department.

There were 20 male and 11 female patients aged 46-79 years (mean: 64.0 years). The cancer-occupied region was located in the cecum in 2 patients, ascending colon in 5, descending colon in 1, sigmoid colon in 7, and rectum in 16. The histological type was well-differentiated adenocarcinoma in 11 patients, moderately differentiated adenocarcinoma in 17, poorly differentiated adenocarcinoma in 1, and mucinous carcinoma in 2.

Methods. Differences in 5-FU sensitivity among types (wild-, homo-, and hetero-types) of each SNP (638G/C, 1050T/A, and 1336A/G) were investigated by the measurement and analytical methods described below.

After informed consent was obtained from the patients, peripheral blood was collected. The protocol was reviewed and approved by the Institutional Review Board of Juntendo University School of Medicine. DNA was extracted from monocytes, and SNPs (638G/C, 1050T/A, and 1336A/G) present in the OPRT coding region were analyzed by the invader assay, in which SNPs were typed by hybridization of allele-specific probes and templates.

Analysis of SNPs in OPRT. The invader assay is capable of analyzing SNP without amplification of nucleic acid (3-5). The procedure is briefly described below: a) signal and invader probes corresponding to each SNP were prepared using probe design software (Invader Creator software, Third Wave Technologies, WC); b) genomic DNA (3 μl, 60 ng/μl)

Table I. Patient background.

No. of patients	31
Median age in years (range)	63 (46-83)
Male/female	20/11
Tumor site (colon/rectum)	15/16
Duke's (A/B/C/D)	0/12/17/2

Table II. Genotype frequency.

No.	638G/C	1050T/A	1336A/G
1	G	T	A
2	G/C	T	A
3	G/C	T	A
4	G	T	A
5	G/C	T	A
6	G	T	A
7	G/C	T/A	A/G
8	G	T	A
9	G/C	T	A
10	G	T	A
11	C	T	A
12	G	T	A
13	G	T	A
14	G	T	A
15	G/C	T/A	A
16	G/C	T	A
17	G	T	A
18	G/C	T	A
19	G	T	A
20	G	T	A
21	C	T	A
22	C	T	A
23	G/C	T	A
24	G	T/A	A
25	G	T	A
26	G	T/A	A/G
27	C	T	A
28	G/C	T/A	A/G
29	C	T	A
30	G/C	T	A
31	G	T	A

was added to a 384-well plate, followed by addition of 6 μl of mineral oil (Sigma Chemical Co., St. Louis, MO), and the plate was heated at 95˚C for 10 min to anneal DNA to single-strands; c) for reaction solution, 1.2 μl of probe mixture, 1.4 μl of FRET solution, and 0.4 μl of cleavase/$MgCl_2$ solution were mixed, and a total volume of 3.0 μl was added to each well. The probe mixture contained the invader probe (0.25 μmol/l), 2 types of signal probe (wild- and mutant-type for detection, 2.5 μmol/l each), and MOPS (10 mmol/l); d) the reaction was performed at 63˚C for 2-4 h in a thermal cycler (PTC-200, MJ Research, Watertown, MA). For detection of fluorescence signals, Cytofluor 4000 (Applied Biosystems, Foster City, CA) was used. The wild-, hetero-, and homo-types were determined based on the signal intensities of FAM dye and Redmond RED dye.

Measurement of 5-FU sensitivity by CD-DST method. 5-FU sensitivity in the tumor region was measured by the CD-DST method reported by Carmichael *et al* (6) and Kobayashi *et al* (7,8): a) the tumor tissue (~1 g) was cut into small pieces and made into a paste. A cell-dispersing enzyme, EZ, was added to the paste, and reacted at 37˚C for 2 h to disperse the cells; b) dispersed cells were collected by centrifugation, and suspended with medium for pre-culture, PCM-1. The cells were pre-cultured in a collagen flask in 5% CO_2 + 95% air for 24 h. After removal of blood cells, necrotized cells, and non-cellular components, collagen gel was solubilized by collagenase solution to suspend viable cells, and the cells were collected by centrifugation; c) the collagen solution and the collected cells were mixed at 1-5x10^5 cells/ml. The mixture was cooled in ice and adjusted to 30 μl/drop, and 3 drops were mounted in each well in a 6-well plate. The plate was incubated at 37˚C in a CO_2 incubator for 1 h to gel the collagen drops; d) the drops were exposed to 5-FU under the following 2 conditions: 10 μg/ml x 3 h, and 0.2 μg/ml x 120 h; e) after the specified time, the medium was aspirated, and the gel drops were washed and cultured in serum-free medium for 7 days; f) viable cells were stained with neutral red and quantified using an imaging analysis system. The colony volume of tumor cells was measured in groups with (T) and without (C) the anticancer drug, and the growth inhibition rate (% IR) was calculated by the equation: [(C-T)/C] x 100.

Results

The backgrounds of the 31 patients are shown in Table I. The genotype frequency is shown in Table II.

No consistent tendency of % IR was noted in any of the wild-, homo-, and hetero-types of 638G/C after exposure at 0.2 μg/ml x 120 h or 10 μg/ml x 3 h (Fig. 2).

As for 1050T/A, the median % IR of the hetero-type was slightly higher than that of the wild-type under both conditions of exposure to 0.2 μg/ml x 120 h and 10 μg/ml x 3 h (Fig. 3). No homo-type of 1050T/A was noted.

As for 1336A/G, the median % IR of the hetero-type was slightly higher than that of the wild-type under both conditions of exposure to 0.2 μg/ml x 120 h and 10 μg/ml x 3 h (Fig. 4). No homo-type of 1336A/G was noted.

Discussion

Although >40 years have passed since the development of 5-FU, it is still the key drug for digestive system cancers, including colon and rectal cancers (9-11).

The action mechanisms of 5-FU are inhibition of thymidylate synthase (TS) by FdUMP and metabolic impairment of RNA by incorporation of FUTP into RNA, and phospho-

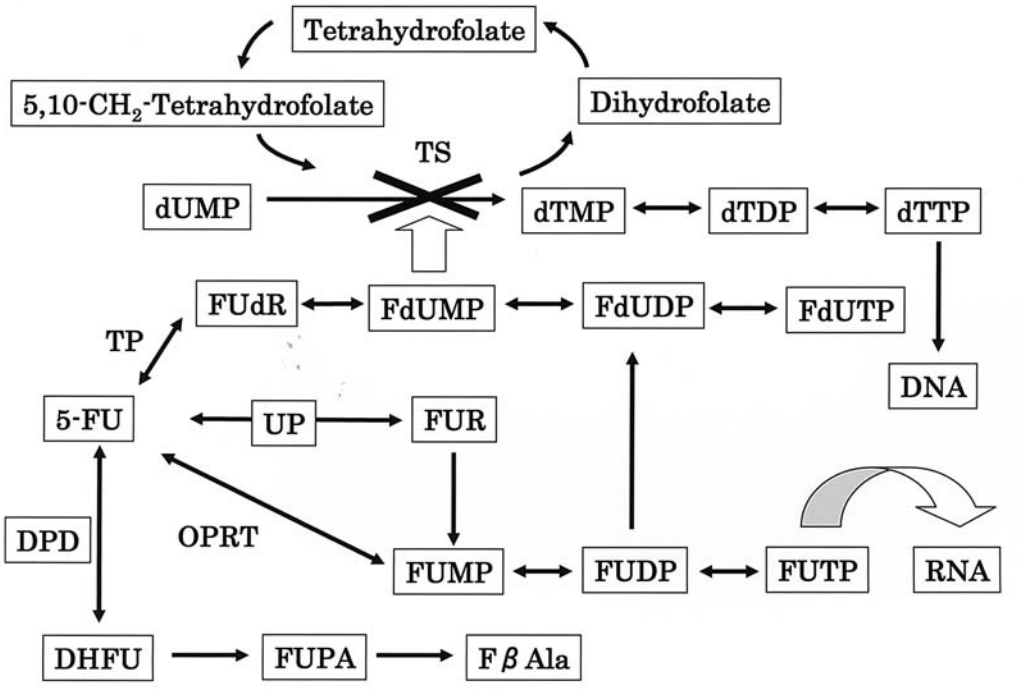

Figure 1. The metabolic change of 5-FU.

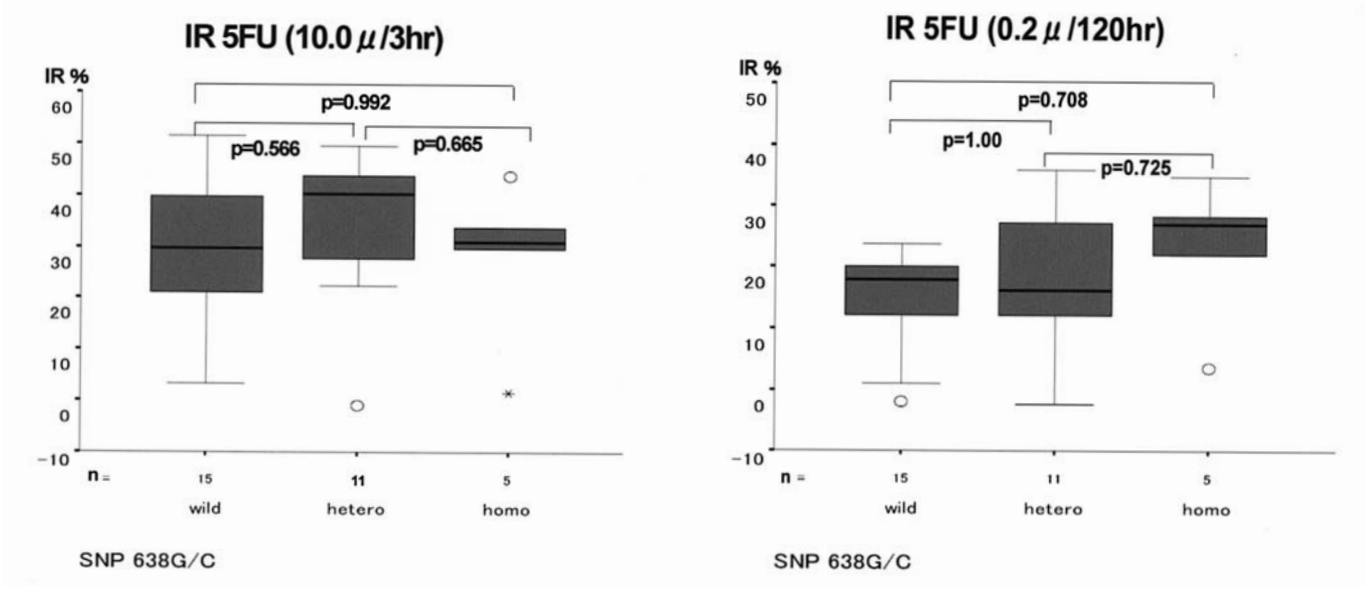

Figure 2. No consistent tendency was noted in % IR of the wild-, homo-, or hetero-type of 638G/C under either exposure condition. Median growth inhibition rates are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and third quartiles. o, outlier; *, extremal value.

rylation of 5-FU is important for both mechanisms (12,13). 5-FU is phosphorylated mainly by 3 pathways, and the phosphorylation pathway by OPRT is considered most important (1). Many studies of chemotherapy mainly using 5-FU for colon and rectal cancers have been performed, and methods expected to increase the effect of 5-FU are performed based on the biochemical modulation theory. However, individual differences are noted in the effect in routine clinical practice, even when the same chemotherapeutic regimen is administered to patients at the same stage. Individual differences in drug effects are noted in not only anticancer drugs including 5-FU but also other drugs, and genetic polymorphism is considered to be a cause because it has a large influence on exertion of effects of drug-metabolizing enzymes that determine the dynamics of drugs in the body (14,15). Genetic polymorphism is defined as the presence of different sequences (substitution, deletion, insertion, and recombination) in a human genomic base sequence at a frequency of 1% or higher. Involvement of

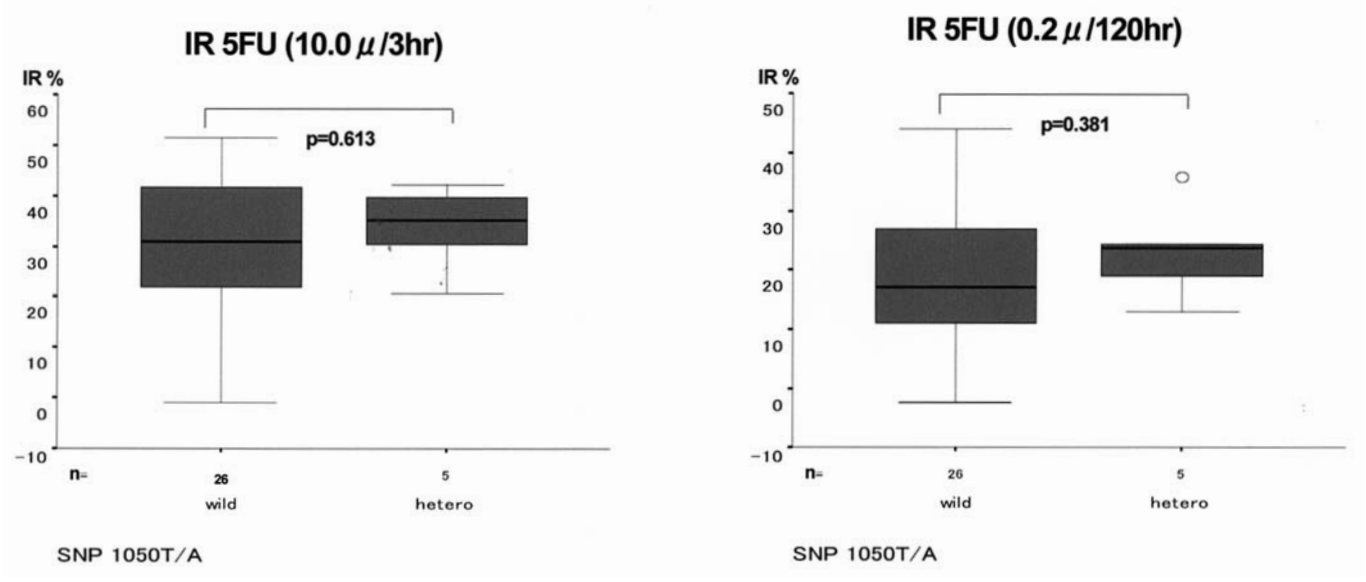

Figure 3. The median % IR was slightly higher in the hetero-type than in the wild-type of 1050T/A under both exposure conditions. Median growth inhibition rates are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and third quartiles. o, outlier.

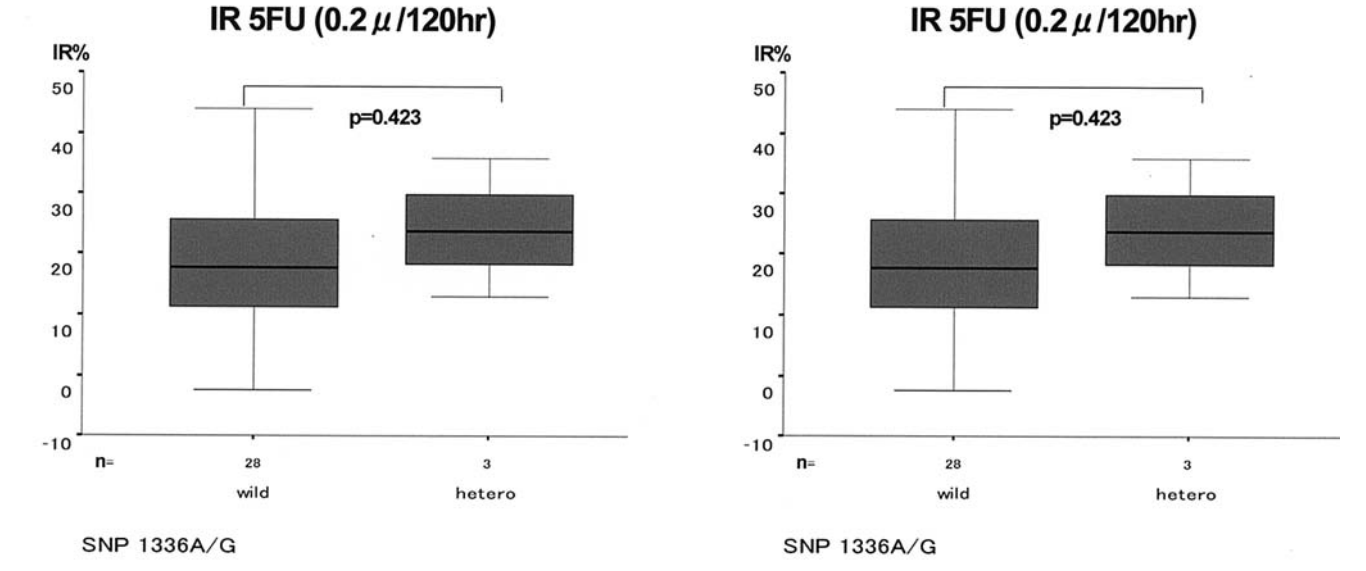

Figure 4. The median % IR was slightly higher in the hetero-type than in the wild-type of 1336A/G under both exposure conditions. Median growth inhibition rates are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and third quartiles.

SNP, in which only 1 nucleotide is different, in sensitivity and adverse effects of various drugs has been elucidated, and its analysis is actively performed (2). SNP is present at 1 in several hundred to several thousand nucleotides in the human genome, and there are >3,000,000 SNPs, accounting for about 85% of genetic polymorphism. We paid attention to the SNP of an enzyme that plays an important role in the metabolic process of 5-FU, OPRT, and investigated whether the presence of SNP causes differences in 5-FU sensitivity of colon and rectal cancers.

SNP analysis of drug metabolism-related enzymes has recently been actively performed, and the presence of 12 SNPs of OPRT has been shown. However, the effects of each SNP on the expression and activity of OPRT have not been clarified, and there has been no report of the relationship with 5-FU sensitivity. Thus, we focused on positions 638 (638G/C) and 1336 (1336A/G) with amino-acid substitutions present in the coding region, which may affect the stereo-structure of OPRT. Regarding silent SNP (sSNP) without amino-acid substitution, such as C3235T of the MDR1 gene, since the presence of sSNP in the coding region affects the exertion of drug effects in some cases, position 1050 (1050T/A) was also analyzed. 5-FU sensitivities of colon and rectal cancers were measured by the CD-DST method developed by Carmichael *et al* (6) and Kobayashi *et al* (7,8). For the exposure conditions, normal clinical conditions, exposure for

me at a low concentration (0.2 μg/ml x 120 h) ıding to continuous intravenous drip infusion, and exposure for a short time at a high concentration (10 μg/ml x 3 h) corresponding to intermittent intravenous bolus injection, were established, and % IR was obtained. Under the above exposure conditions, differences in sensitivity among the types (wild-, homo-, and hetero-types) were investigated in each SNP. However, no obvious relationship was noted between SNP at the 3 sites and 5-FU sensitivity. Although the number of patients analyzed was small, the results may have represented the analysis of 3 of the 12 SNPs. For example, it is possible that regulatory SNP (rSNP) is present in the promoter region and affects the protein expression level, as 308G/A in TNFα, or that intron SNP (iSNP) is present in the regulatory region and affects the phenotype. Thus, analysis of the SNP of OPRT present in regions other than the coding region is necessary. Since the median of 5-FU sensitivity was slightly higher in the hetero-type than in the wild-type of 1050T/A and 1336A/G, analysis of additional patients may provide some information.

Linkage disequilibrium of some SNPs has been shown, in which SNPs are linked in expression at a high frequency and affect enzyme activity, and some cases have been explained mostly with several alleles (16). It is difficult to discuss this point in this study, but if linkage disequilibrium is noted in the SNP of OPRT, consideration of haplotypes, which are allele combination patterns of SNP, is necessary for analysis. No obvious relationship was noted between the 3 selected sites of SNP and 5-FU sensitivity, but many points remain unclear in the relationship between the SNP of OPRT and 5-FU sensitivity. Analysis of all these points by detailed investigation may provide new information for chemotherapy using 5-FU as the key drug.

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