predicted three-dimensional structural model of the MLH1 NH_2-terminal domain by a homology modeling method to map variants in the predicted structure (Fig. 4*A–C*). Most of the variants predicted to be around the ATP-binding pocket were DME negative in at least one of the yeast assays or MMR− (Fig. 4*B*). In particular, variants mutated in residues thought to be critical for ATP binding, such as M35R, N38D, S44F, G67R, I68N, C77R, F80V, T82I, K84E, R100P, and I107R, affected both DME and *in vitro* MMR activity (Fig. 4*C*). Unlike the NH_2-terminal ATPase domain, sequence identity among the COOH-terminal domains of MutL homologues is not high enough to construct three-dimensional structure by homology modeling; however, the detailed analysis of secondary structure predictions and sequence alignments indicates that the structures of the COOH-terminal domains of MutL homologues are similar (Fig. 4*D*; refs. 11, 35). Missense variants were classified by the assays and mapped on a COOH-terminal domain alignment of four MutL homologues, showing that variants in the COOH-terminal domain were found more frequently in the internal subdomain, especially within and around the αC helix, than in the external subdomain and functionally inactive variants were distributed through the whole region of the COOH terminus (Fig. 4*D*).

Relations between MLH1 functions and clinical features. To investigate the relationship between the functional phenotype in the assays and clinical phenotypes previously reported in the databases or papers, at first, *in vitro* MMR activities were compared between AC+ variants and AC− variants, considering the total number of families reported for each variant. The AC+ group showed significantly lower *in vitro* MMR activities than AC− group ($P < 0.01$; Fig. 5*A*). Second, the MLH1 protein expression levels in HCT116 cells were compared between AC+ and AC− groups. The significant difference of the protein level was not shown between the two groups ($P = 0.68$; Fig. 5*B*). These results suggested that there is correlation between MMR defects and a strong family history but no correlation between the MLH1 protein instability and a strong family history.

Discussion

In this study, we evaluated the functional significance of 101 *MLH1* variants by yeast-based assay and *in vitro* assay to provide useful information for understanding of pathogenicity. This functional characterization of a large number of variants allowed us to compare the property of two assays and to analyze the structural basis of functional deficiency.

We showed that the yeast assay distinguished the majority of variations that retained or lost the *in vitro* MMR activity. These data confirmed the accuracy and usefulness of the yeast assay as a simple method having an advantage to analyze a large number of variations without laborious steps. The three kinds of reporter systems identified some variants showing different DME phenotype among yeast assays (DME1+ or DME2+), although the majority (75 of 101, 74.3%) of the *MLH1* variants showed consistent DME (DME− or DME3+). These discrepant variations showing DME1+ or DME2+ were supposed to be functionally subtle because these difference could be explained by the distinct thresholds of the reporter genes (*LacZ*, *GFP*, or *ADE2*), the target nucleotide repeats (mono- or di-), and/or the location of the repeats (a plasmid or a chromosome). This is supported by our finding that the average values of the *in vitro* MMR activities depended on the degree of the DME (from DME− to DME3+; Fig. 3*A*). Thus, a combination of the three yeast assays has the ability to evaluate functionally subtle variants as well as the *in vitro* MMR assay. Among variants showing discrepant results between the yeast assays and *in vitro* MMR assay, variants showing both DME3+ in the yeast assays and MMR− in the *in vitro* MMR assay (DME3+/MMR−) should be estimated to be pathogenic because MMR deficiency should link directly with carcinogenesis by causing genome instability regardless of the DME in yeast. DME−/MMR+ variants are difficult to be interpreted but possibly defect some unknown function in human cells, because the phenotype of these variants in yeast cells are quite different from that of a wild-type. We consider that DME1+ or DME2+ variants can also lose some function partially for the same reason.

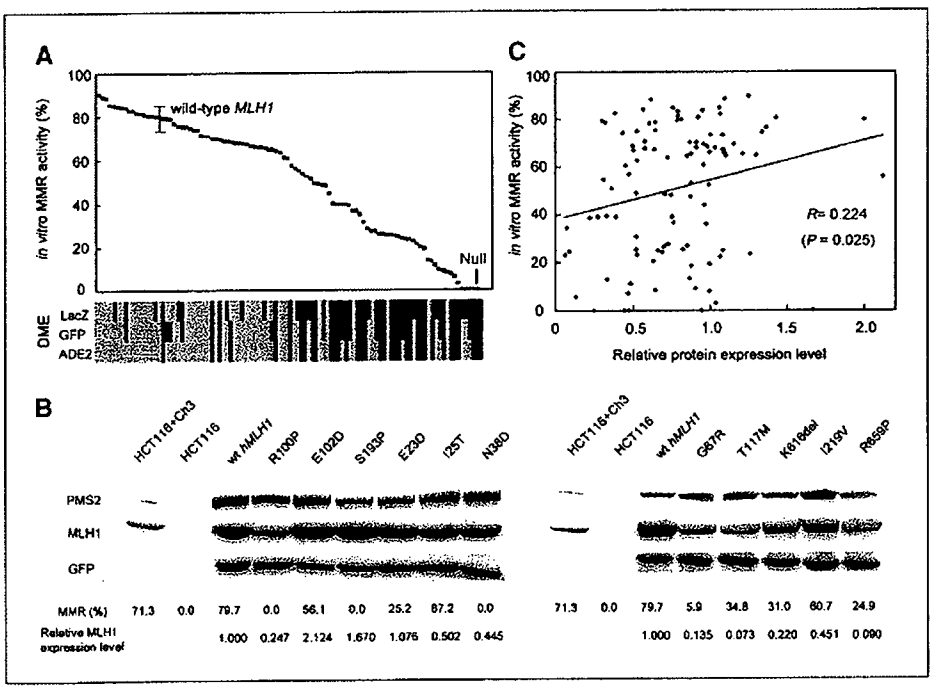

Figure 2. Protein levels of expressed MLH1 and functional assays. *A*, *top*, *in vitro* MMR activities of the 101 *MLH1* variants in order of their value. *Points*, mean MMR activity of wild-type MLH1; *bars*, SD. *Bottom*, the corresponding DME status. *Gray box*, DME positive; *black box*, DME negative. *B*, Western blot analyses of 11 representative MLH1 variants coexpressed with PMS2 in HCT116 cells. MLH1 protein levels were normalized by the coexpressed GFP level. The ratios of the variants to the wild-type were shown as the relative MLH1 protein level. The *in vitro* MMR activities of cell extracts expressing MLH1 variants are also shown below. *C*, correlation between *in vitro* MMR activities and the relative protein levels of 101 MLH1 variants. Their relationship was investigated by Pearson's correlation coefficient test.

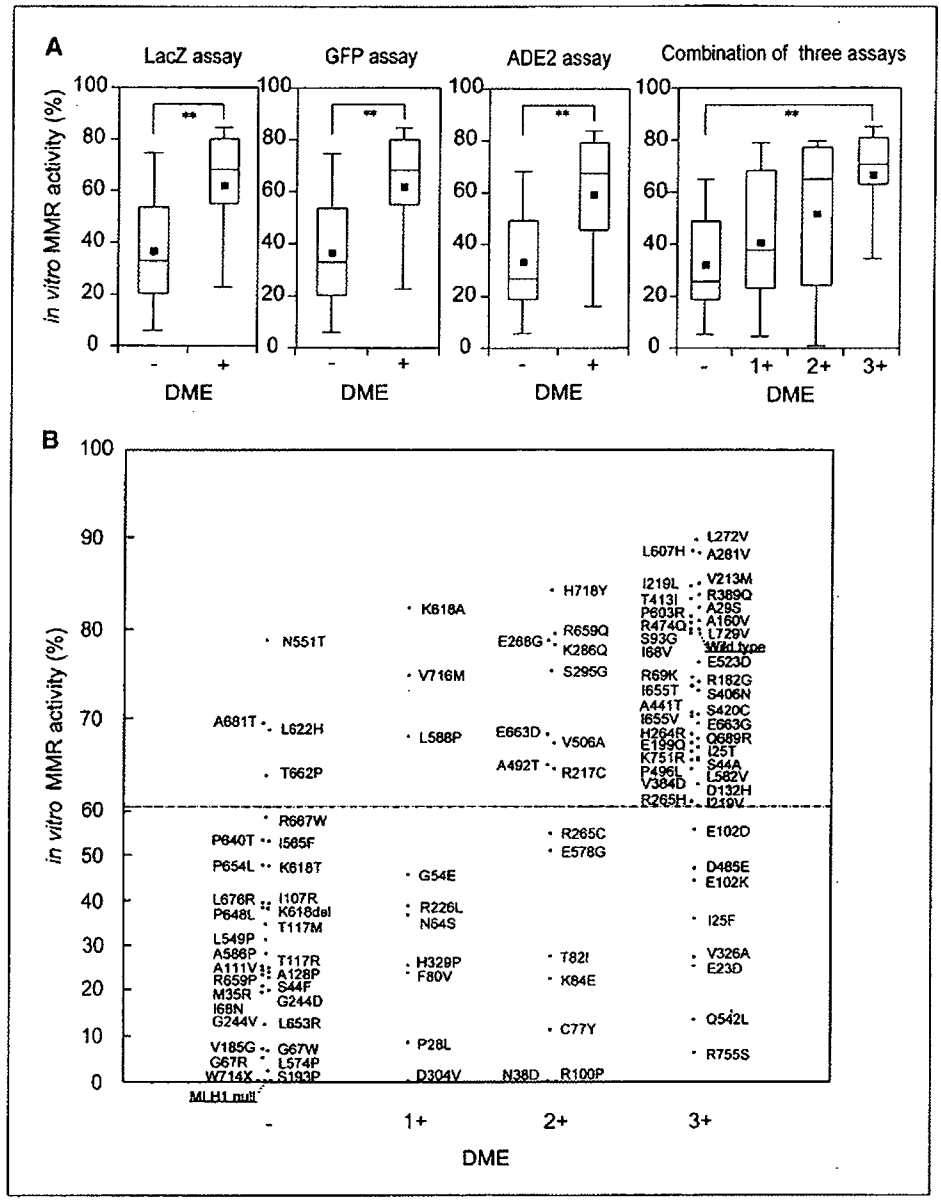

Figure 3. Comparison of the DME of yeast assays with the *in vitro* MMR activity. *A*, box-and-whisker plot of *in vitro* MMR activity in DME-negative and DME-positive *MLH1* variants. *Horizontal line inside the box*, median. *Upper and lower limits of the box*, 75th and 25th percentiles, respectively. *Horizontal bars above and below the box*, 90th and 10th percentiles, respectively. ■, mean of data points. **, $P < 0.01$, two-sided Mann-Whitney *U* test. *B*, functions of *MLH1* variants in an *in vitro* MMR assay and yeast assays. Wild-type *MLH1* and 101 variants are plotted by *in vitro* MMR activity and DME level. *Dotted line*, *in vitro* MMR activity of common polymorphism I219V.

Other functional analyses examining a part of 101 variants agree with our functional evaluation (2–5, 7). For example, 34 *MLH1* variants were recently analyzed for four distinct functional properties containing *in vitro* MMR assay, protein expression, protein localization, and interaction with PMS2, and were evaluated for their pathogenicity by integrating all of the results (8). Among 20 variants assayed in common with this study, the *in vitro* MMR activities were low (<48.7% activity) in their 12 pathogenic variants, and high (>60.7%) in their eight nonpathogenic variants. DME phenotype in this study also corresponded with their interpretation in 16 of 20 variants. The consistencies in the functionally known *MLH1* variants supported reliability of the functional evaluation for the newly analyzed variations in this study.

Our final purpose of analyzing the large number of *MLH1* variants is to establish a database for understanding the pathogenicity of the gene alterations. One of the major problems in using our data for clinical purpose is that the appropriate cutoff value is difficult to set up, because we cannot estimate how intermediate or subtle functional defects contribute to the pathogenesis in HNPCC. However, the information on common polymorphisms found in the normal populations can be useful to consider the tolerable level predicted to be functionally proficient. The most common polymorphism is I219V, which was reported from various countries, although the allele frequency is varied from 3% to 36% (24, 38, 39). According to our data, I219V retained ~60% of MMR activity and DME3+. The other seven putative polymorphisms retained also >60% MMR activity, the seven of the eight showed DME3+ except H718Y showing DME2+. Therefore, we currently propose that both DME3+ and 60% of MMR activity is the reasonable cutoff value to estimate the variations not associated with pathogenicity in HNPCC. Then, 50 *MLH1* variations with more than this MMR activity are categorized into MMR+ ones, and 35 DME3+/MMR+ variants are thought to be functionally proficient. The D132H also retains DME3+/MMR+, which is a polymorphism

among Israeli populations not associated with HNPCC but the sporadic colorectal cancer predisposition (23). Thus, we cannot exclude the possibility that even the variations showing DME3+/MMR+ might be involved in the sporadic carcinogenesis.

Another critical problem is that amino acid substitutions in MLH1 affected both protein expression levels and functions. Our data showed that there was just a slight correlation between MLH1 protein levels and *in vitro* MMR activities (Fig. 2*C*). Therefore, we predict that there are at least two mechanisms inactivating MLH1 function by amino acid substitutions, the shortage of MLH1 protein by protein instability, and functional inactivation by structural alteration. This suggested that the cutoff values are required in both functional level and protein level for overall evaluation. Recent studies have indicated that the immunohistochemical analysis of MMR proteins is one of the most efficient and sensitive screening method to detect abnormalities in MMR proteins (40, 41). In our data, *in vitro* MMR activities were impaired without reducing MLH1 protein levels in some *MLH1* missense variants. This observation suggested that these pathogenic variants can be detected positively by immunohistochemistry, although we cannot

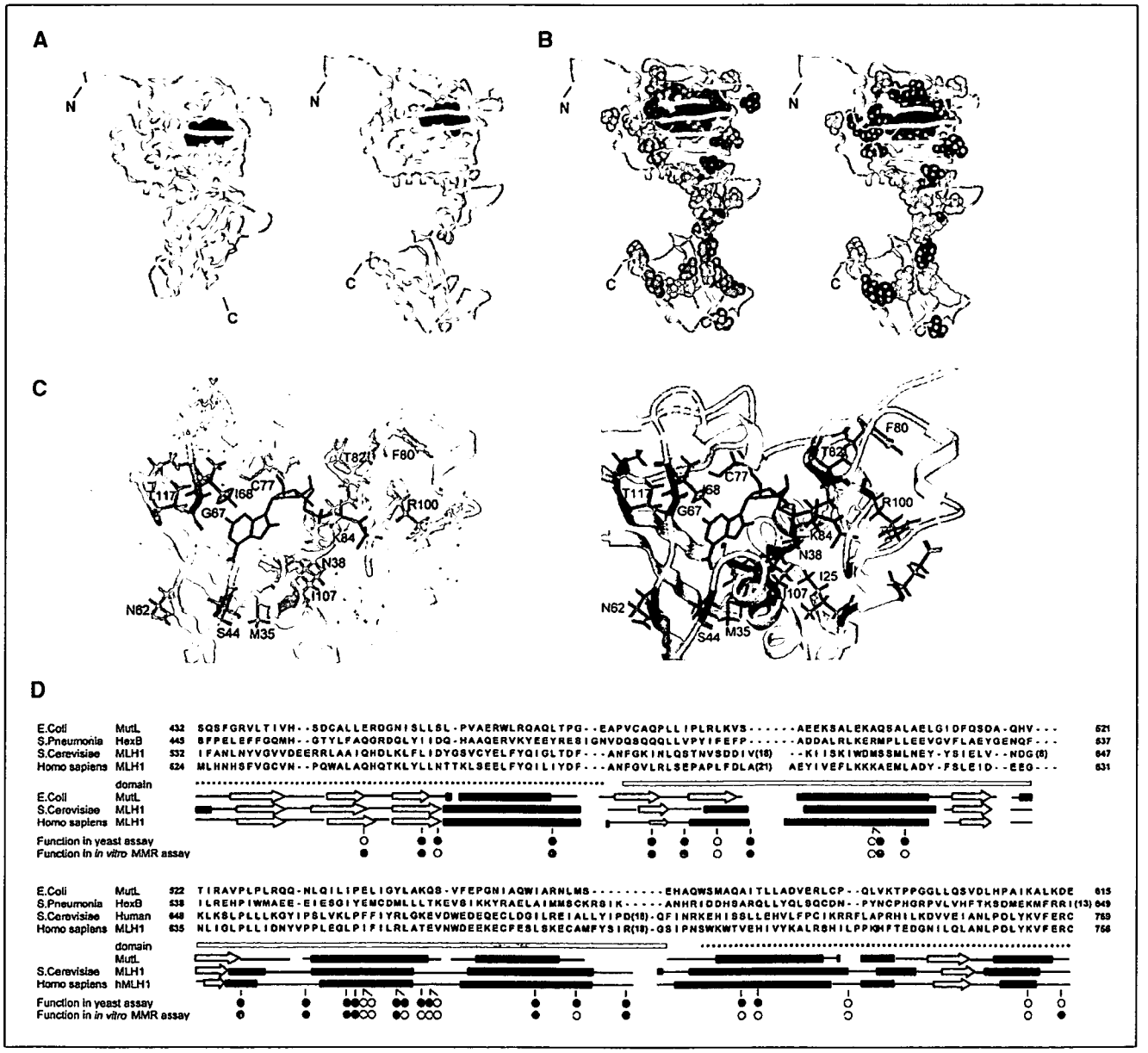

Figure 4. Relationships between putative MLH1 protein structures and functions. *A* to *C*, model of the three-dimensional structure of the MLH1 NH_2-terminal domain and maps of the MLH1 variants. *A*, ribbon diagram of the *E. coli* MutL NH_2-terminal domain (*left*) and an MLH1 NH_2-terminal domain simulated by homology modeling (*right*). *B* and *C*, the model of the whole of the MLH1 NH_2-terminal domain and the model of the ATPase domain, respectively. *Colored balls or bars*, amino acid residues examined in this study and the functional information from yeast assays and *in vitro* MMR assay. *Red*, *pink*, and *flesh color*, DME−, DME1+/2+, and DME 3+ phenotypes, respectively. *Green*, *light green*, and *yellow*, higher (≥75% of wild-type), average (50–75%), and (<50% of wild-type) lower *in vitro* MMR activity, respectively. *Blue balls or bars*, ADPnP. *D*, sequence alignment and secondary structure of the COOH-terminal domains of MutL homologues. *Arrows* and *bars*, β-sheet and α-helix, respectively. *Regular lines*, internal subdomain; *dotted orange lines*, external subdomain. *Colored dots*, locations of MLH1 variants, representing the functional phenotype, as in (*B*) and (*C*).

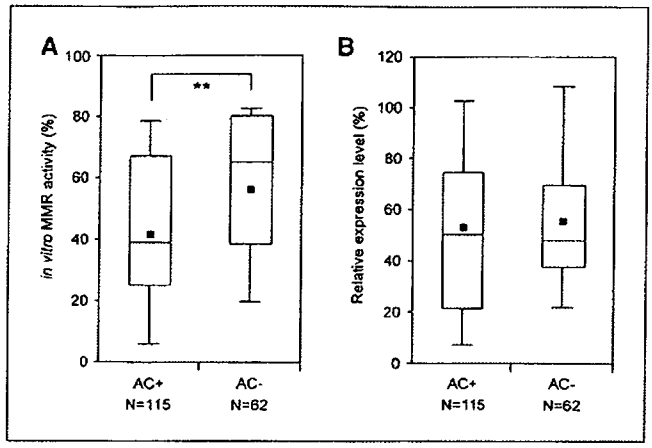

Figure 5. Relationships between the functions and clinical features of *MLH1* variants. *A*, box-and-whisker plot of *in vitro* MMR activity of *MLH1* variants from families fulfilling the Amsterdam criteria (AC+) or not (AC−). *B*, box-and-whisker plot of the protein expression levels of the AC+ or AC− variants. *Line inside the box*, median. *Upper and lower limits of the box*, 75th and 25th percentiles, respectively. *Vertical bars above and below the box*, 90th and 10th percentiles, respectively. ■, mean of data points. **, $P < 0.01$ by two-sided Mann-Whitney *U* test.

directly link the protein amount of transient expression in cell lines with the endogenous protein level in tumors of the corresponding mutation carrier. Among the 50 variants with MMR−, 20 (40%) retained >75% of the MLH1 expression level of a wild-type. We speculate that these 20 variants (E23D, I25F, P28L, G54E, N64S, F80V, T82I, K84E, E102D, E102K, S193P, G244V, D304V, H329P, Q542L, I565F, L574P, P640T, and R755S) will be the candidate pathogenic variants with difficulty in clear detection as loss of protein expression by immunohistochemistry. Among them, three variants, P28L, F80V, and P648L, actually have been shown to retain their protein levels by immunohistochemical analyses (8, 37). Functional assays are especially useful for these variants because the abnormality will not be detected by immunohistochemistry until subsequent sequence analysis and functional assays.

Several lines of biochemical investigations have shown that the heterodimerization with counterpart proteins such as PMS2 and conformational change by ATP binding are important in the MLH1 function (1, 3, 7–10). Mapping 101 *MLH1* variants on *MLH1* cDNA indicated that the majority of functionally defective *MLH1* variants (showing DME−, DME1+, or DME2+ and/or MMR−) were located within two functional domains, the NH_2-terminal ATPase and the COOH-terminal PMS2-interactive domains. In particular, almost all variants involved in the ATP-binding pocket were functionally defective (Fig. 4*B* and *C*). The homologous structure of the NH_2-terminal *E. coli* MutL provided molecular basis for functional defects of human *MLH1* missense variants (9, 10). P28L, M35R, and S44F can disrupt ATP binding and hydrolysis; G67R, I68N, I107R, T117M, and T117R degraded ATP binding pocket; and other variants (A128P, V185G, R226L, G244D, and V326A) can alter or destabilize the overall protein folding. Our functional assay showed that 13 out of these 14 variants were functionally defective and supported the functional prediction based on the protein structure. In contrast, some variants probably do not change the protein structure because the changes are conservative (I25F, A29S, S44A, I68V, V213M, and I219L; refs. 9, 10). Recent study resolved crystal structure of COOH-terminal MutL and identified dimerization interface (11). The alignment and homology modeling show that L653R, R654L, and R659P resided on the equivalent to the dimmer interface of MutL, which is the putative interface of MLH1 with PMS2. T662P is equivalent site of MutL critical for binding with DNA (Fig. 4*D*). The homology modeling provides the structural basis of functional deficiency for some variants, although the functionally deficient variations of *MLH1* distributed through the whole COOH terminus.

The previous studies have shown that *MSH6* mutations cause a partial MMR deficiency and related with atypical HNPCC families (42). This suggests the possibility that intermediate functional deficiency is associated with the atypical HNPCC kindred with weak family history or late onset. Based on this hypothesis, one of the methods to validate our functional assay is investigating the relationship between functional evaluations with clinical feature such as family history. The median MMR activity was significantly lower among the AC+ families (38.9%) than the AC− families (65.1%), whereas the median level of relative protein expression has no significant difference between these two groups (Fig. 5). Therefore, AC status has correlation with our functional data at least stronger than with protein level. Functions in the assays well correlated with clinical features, whereas it can be said that the Amsterdam criteria links with functions well and is a very useful clinical diagnostic criteria. Our functional data have consistency with previous functional assays in a majority of the 45 functionally known variants, including some variants evaluated for pathogenicity by both cosegregation study and functional assay. Precise family information was described even in the limited number among some of the variants newly analyzed in this study. For example, S193P was detected in three affected members and two other members among 12 individuals in an AC+ family (24). This variant showed DME−/MMR− in our assays and then indicated to be pathogenic from both family history and functional assay. R687W was found in three affected members but not two healthy members in the AC+ family, suggesting that this variant is likely pathogenic (20). However, our functional data indicated DME− but relatively high MMR activity (57.2%) even below 60% (defined to be MMR− in this study). Yeast assay detected pathogenicity of this variant more clearly than MMR in this case. Although L607H was found in one family member with colon cancer but not in two healthy members in the family, this colon cancer did not show any microsatellite instability, low staining by immunohistochemistry (29), or functional defect in our assay. Then, pathogenicity of this variant is supposed to be in question but functional data correlates with microsatellite instability and immunohistochemical results. Thus, in the several cases in which detailed clinical information is available, the functional phenotype well correlated with clinical features for families carrying the corresponding variants. Based on this, it may be expected that the pathogenicity can be well predicted by the functional phenotype in the assay for many of the other variants, especially those showing clear phenotype such as DME−/MMR− and DME3+/MMR+. However, we have to accumulate more data on clinical and functional characteristics and evaluate accurate sensitivity and specificity of functional data for predicting pathogenicity, to use for practical purposes in clinics, for example, to decide whether surveillance of a mutation noncarrier should be continued.

In this study, we analyzed functional significances of 56 *MLH1* variants that have never been evaluated in any functional assay. Without functional analyses, pathogenicity of missense variants are estimated usually based on amino acid property, conservation

among species, or computed prediction like SIFT as a recent way. The SIFT has a good accuracy for the functional prediction; however, ~20% of variants could fail to be predicted. A similar tendency has been observed in another analysis (8) and in other proteins such as p53 tumor-suppressor protein (data not shown). Therefore, it is still desirable to carry out biochemical assays to predict the pathogenicity for variants newly reported if available.

In summary, we examined a large number of *MLH1* variants using both yeast and *in vitro* functional assays and characterized the functional alterations of the variants. We confirmed that the majority of functionally inactive variants were located in the NH_2-terminal and COOH-terminal domains, especially around the ATP-binding pocket and the region responsible for heterodimerization with other MutL homologues. The results corresponded well with observations by the structural analysis of *E. coli* MutL crystals. The study described here should be useful for evaluating cancer risks in individuals or families carrying *MLH1* variants and may provide clues for better understanding MLH1 functions.

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Review Article

Genes Regulating the Sensitivity of Solid Tumor Cell Lines to Cytotoxic Agents: A Literature Review

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In order to review gene alterations associated with drug responses *in vitro* to identify candidate genes for predictive chemosensitivity testing, we selected from literature genes fulfilling at least one of the following criteria for the definition of '*in vitro* chemosensitivity associated gene': (i) alterations of the gene can be identified in human solid tumor cell lines exhibiting drug-induced resistance; (ii) transfection of the gene induces drug resistance; (iii) down-regulation of the gene increases the drug sensitivity. We then performed Medline searches for papers on the association between gene alterations of the selected genes and chemosensitivity of cancer cell lines, using the name of the gene as a keyword. A total of 80 genes were identified, which were categorized according to the protein encoded by them as follows: transporters ($n = 15$), drug targets ($n = 8$), target-associated proteins ($n = 7$), intracellular detoxifiers ($n = 7$), DNA repair proteins ($n = 10$), DNA damage recognition proteins ($n = 2$), cell cycle regulators ($n = 6$), mitogenic and survival signal regulators ($n = 7$), transcription factors ($n = 4$), cell adhesion-mediated drug resistance protein ($n = 1$), and apoptosis regulators ($n = 13$). The association between the gene alterations and chemosensitivity of cancer cell lines was evaluated in 50 studies for 35 genes. The genes for which the association above was shown in two or more studies were those encoding the major vault protein, thymidylate synthetase, glutathione S-transferase pi, metallothionein, tumor suppressor p53, and bcl-2. We conclude that a total of 80 *in vitro* chemosensitivity associated genes identified in the literature are potential candidates for clinical predictive chemosensitivity testing.

Key words: chemotherapy sensitivity drug resistance solid tumor

INTRODUCTION

Malignant neoplastic diseases remain one of the leading causes of death around the world despite extensive basic research and clinical trials. Advanced solid tumors, which account for most malignant tumors, still remain essentially incurable. For example, 80% of patients with non-small cell lung cancer have distant metastases either at the time of the initial diagnosis itself or at the time of recurrence after surgery for the primary tumor. Systemic chemotherapy against malignant tumors remains of limited efficacy in spite of the development in the recent past of several new chemotherapeutic agents; therefore, patients with distant metastases rarely live for long (1).

Tumor response to chemotherapy varies from patient to patient, and clinical objective response rates to standard chemotherapeutic regimens have been reported to be in the range of 20–40% for most common solid tumors. Thus, it would be of great benefit it became possible to predict chemosensitivity of various tumors even prior to therapy. DNA, RNA and protein-based chemosensitivity tests have

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been performed in an attempt to predict the clinical drug response, but the precise gene alterations that might be predictive of the chemosensitivity of the tumors are still unknown. Here we aimed to review the gene alterations that may be associated with the drug response *in vitro* (*in vitro* chemosensitivity associated genes) in order to identify candidate genes for predictive chemosensitivity testing in the clinical setting. The association between these gene alterations and clinical chemosensitivity in lung cancer patients has been reported elsewhere (2).

METHODS

In vitro chemosensitivity associated genes were identified from the medical literature as described previously (2). Briefly, we conducted a Medline search for papers on tumor drug resistance published between 2001 and 2003. This search yielded 112 papers, including several review articles. Manual search of these papers led to identification of 134 genes or gene families that were potentially involved in drug resistance based on their function. We conducted a second Medline search for *in vitro* studies of the 134 genes or gene families using the name of the gene as a keyword. Genes that fulfilled at least one of the following criteria for the definition of *in vitro* chemosensitivity associated gene were selected from the 134 genes: (i) alterations of the gene can be identified in a human solid tumor cell lines exhibiting drug-induced resistance; (ii) transfection of the gene induces drug resistance; (iii) down-regulation of the gene or of the protein encoded by it increases the drug sensitivity. For this last category, we included studies in which the gene expression or function was suppressed by antisense RNA, hammerhead ribozyme, or antibody against the gene product. Finally, a Medline search for papers on the association between gene alterations and chemosensitivity of solid tumor cell lines was performed using the name of the gene as a keyword. Papers in which the association was evaluated in 20 or more cell lines were included in this study. The name of each gene was standardized according to the Human Gene Nomenclature Database of National Center for Biotechnology Information (NCBI).

RESULTS

Of the 134 genes or gene families, gene alterations were found in cells exhibiting drug-induced resistance, transfection of the gene increased or decreased the drug resistance,

Table 1. Transporters and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
ABCA2	U		S	Estramustine		1
ABCB1	U	R	S	DOX, PTX, VCR, VBL	Yes (lung, DOX)	2 11
					No (lung, DOX)	12
ABCB11		R		PTX		13
ABCC1	U	R	S	CPT, DOX, ETP, MTX, VCR	Yes (lung, CDDP, DOX)	11,14 21
					No (lung, PTX)	22
ABCC2	U	R	S	CDDP, DOX, MTX, VCR	No (lung, DOX)	18, 21, 23 25
ABCC3	NC, U	R		ETP, MTX	Yes (lung, DOX)	21, 25 28
ABCC4	NC, U	NC, R		MTX	No (lung, DOX)	12, 25, 29 31
ABCC5	NC, U	NC		DOX, MIT	Yes (lung, ETP)	12 25, 31 34
ABCG2	M, U	R		DOX, MIT, MTX, SN38, TOP		35 43
MVP	U		NC	DOX	Yes (brain, CDDP, DOX)	44 47
					Yes (lung, DOX)	10
ATP7A	U			CDDP		48
ATP7B	U	R		CDDP		48 52
SLC29A1	U			5-FU	No (NCI-panel)	52, 53
SLC28A1		S		5′-DFUR	No (NCI-panel)	53, 54
SLC19A1	D	S		MTX	Yes (NCI-panel)	55 58

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; M, mutated; NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): NC, no change; R, resistant; S, sensitive

Drugs: CDDP, cisplatin; CPT, irinotecan; DOX, doxorubicin; ETP, etoposide; MIT, mitoxantrone; MTX, methotrexate; PTX, paclitaxel; SN38, irinotecan metabolite; TOP, topotecan; VBL, vinblastine; 5-FU, 5-fluorouracil, 5′-DFUR, 5′-deoxy-5-fluorouridine, capecitabine metabolite

Table 2. Drug targets, the associated proteins, and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
TUBB	IEC, M			PTX		59–63
TUBB4	U		S	PTX	Yes (NCI-panel, PTX)	59, 60, 63–66
TUBA	IEC, M	R		PTX		64, 67, 68
TYMS	U	R	S	5-FU	Yes (renal cell, 5-FU)	69–74
					No (NCI-panel, 5-FU)	75
					Yes (lung, DOX)	10
TOP1	M	R*		CPT		76–84
TOP2A	M, D			ETP, DOX	No (lung, DOX)	10, 82–91
TOP2B	D			ETP		86, 87
DHFR	M, U	R*		MTX		92–96
MAP4		S		PTX		97
MAP7		S		PTX		98
STMN1	U	R		PTX		99, 100
KIF5B		R	R	ETP, PTX		101, 102
HSPA5		R		ETP		103
PSMD14		R		CDDP, DOX, VBL		104
FPGS	D			5-FU		105

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; IEC, isoform expression change; M, mutated; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): R, resistant; S, sensitive. Drugs: CDDP, cisplatin; CPT, irinotecan; DOX, doxorubicin; ETP, etoposide; MTX, methotrexate; PTX, paclitaxel; VBL, vinblastine; 5-FU, 5-fluorouracil.
*Over-expression of the mutant gene.

and down-regulation of the gene altered the drug sensitivity for 45, 57 and 32 genes, respectively, and a total of 80 genes fulfilled the criteria for the definition of an '*in vitro* chemosensitivity associated gene'. The genes were categorized according to the protein encoded by them as follows: transporters ($n = 15$, Table 1), drug targets ($n = 8$, Table 2), target-associated proteins ($n = 7$, Table 2), intracellular detoxifiers ($n = 7$, Table 3), DNA repair proteins ($n = 10$,

Table 3. Intracellular detoxifiers and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
GSTP1	U		S	CDDP, DOX, ETP	Yes (lung, DOX)	10, 106, 107
					Yes (NCI-panel)	108
GPX		R, NC		DOX	Yes (lung, CDDP)	109–112
GCLC	U	R	S	CDDP, DOX, ETP	Yes (NCI-panel)	106, 108, 113–121
GGT2	U	R		CDDP, OXP		114, 117, 122, 123
MT	U, NC	R		CDDP	Yes (urinary tract, CDDP)	118, 124–130
					Yes (lung, DOX)	10, 131
RRM2	U	R		5-FU, GEM, HU		71, 132–134
AKR1B1	U			DNR		135

Alterations in drug-induced resistance cells (DIRC): NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): NC, no change; R, resistant; S, sensitive. Drugs: CDDP, cisplatin; DNR, daunorubicin; DOX, doxorubicin; ETP, etoposide; GEM, gemcitabine; HU, hydroxyurea; OXP, oxaliplatin; 5-FU, 5-fluorouracil.

Table 4. DNA damage recognition and repair proteins and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
HMGB1	U			CDDP		136
HMGB2		S		CDDP		137
ERCC1	U	R	S	CDDP		138–140
XPA	U	R		CDDP	No (NCI-panel)	141–143
XPD		R		CDDP	Yes (NCI-panel)	142–144
MSH2	D, NC			CDDP		145, 146
MLH1	D, NC			CDDP		145–147
PMS2	D, NC			CDDP		146, 147
APEX1		R		BLM		148
MGMT		R	S	CPM, ACNU	Yes (lung, DOX)	10, 149–152
BRCA1	U	S	R	PTX		153–155
GLO1		R		DOX		156

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): R, resistant; S, sensitive. Drugs: ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea; BLM, bleomycin; CDDP, cisplatin; DOX, doxorubicin; PTX, paclitaxel.

Table 4), DNA damage recognition proteins ($n = 2$, Table 4), cell cycle regulators ($n = 6$, Table 5), mitogenic and survival signal regulators ($n = 7$, Table 6), transcription factors ($n = 4$, Table 6), cell adhesion-mediated drug resistance protein ($n = 1$, Table 6), and apoptosis regulators ($n = 13$, Table 7).

The association between the gene alterations and *in vitro* chemosensitivity was evaluated in one study for 25 genes, in two studies for seven genes, in three studies for two genes, and in five studies for one gene, and in a total of 50 studies for 35 genes (Table 8). Significant association was found between chemosensitivity and alterations of genes encoding transporters, drug targets and intracellular detoxifiers (Table 8). Genes for which such association was shown in two or more studies were those encoding the major vault protein/lung resistance-related protein (*MVP*) (Table 1), thymidylate synthetase (*TYMS*) (Table 2), glutathione S-transferase pi (*GSTP1*), metallothionein (*MT*) (Table 3), tumor suppressor protein p53 (*TP53*), and B-cell CLL/lymphoma 2 (*BCL2*) (Table 7).

DISCUSSION

We identified a total of 80 *in vitro* chemosensitivity associated genes. These genes have been the subject of considerable research, and of numerous scientific publications. In addition, we may also have to expect the existence of many other genes associated with chemosensitivity

Table 5. Cell cycle regulators and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
RB1		R		DOX	Yes (lung, DOX)	157–159
					No (lung, CDDP, DOX)	160
GML		S		MMC, PTX	Yes (lung, CDDP)	161–163
CDKN1A	U	R, S	S	CDDP, BCNU, PTX		164–171
CCND1		R, S	S	CDDP, MTX, PTX	No (lung, DOX)	10, 172–176
CDKN2A		S, R		CDDP, 5-FU, PTX, TOP	Yes (brain, 5-FU)	177–184
CDKN1B		R		DOX		185

Alterations in drug-induced resistance cells (DIRC): U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): R, resistant; S, sensitive. Drugs: BCNU, carmustine; CDDP, cisplatin; DOX, doxorubicin; MMC, mitomycin C; MTX, methotrexate; PTX, paclitaxel; TOP, topotecan; 5-FU, 5-fluorouracil

Table 6. Mitogenic and survival signal regulators, integrins, transcription factors and *in vitro* evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no
		UCs	DCs			
ERBB2		R, NC	S	CDDP, PTX	Yes (lung, DOX)	10, 22, 186–191
EGFR		R		DOX	No (lung, CDDP, DOX, PTX)	10, 22, 112, 192
KRAS2		R*		CDDP		193
HRAS		R*, NC		Ara-C, DOX, PTX	No (lung, DOX)	10, 193–197
RAF1		R		DOX		198
AKT1		NC, R	S	CDDP, DOX, PTX		199–201
AKT2		R	S	CDDP		200, 202
ITGB1			S	ETP, PTX		203, 204
JUN		R		CDDP	No (lung, DOX)	10, 205
FOS	U	R	S	CDDP	No (lung, DOX)	10, 206–208
MYC	NC, U	S, R	R, S, NC	CDDP, DOX	No (lung, DOX)	10, 209–216
NFKB1	U		S	5-FU, DOX, ETP		217–222

Alterations in drug-induced resistance cells (DIRC): NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs)): NC, no change; R, resistant; S, sensitive. Drugs: Ara-C, 1-beta-D-arabinofuranosylcytosine; CDDP, cisplatin; DOX, doxorubicin; ETP, etoposide; PTX, paclitaxel; 5-FU, 5-fluorouracil.
*Up-regulated with mutated K-ras gene.

Table 7. Apoptosis regulators and in vitro evidence of association with chemosensitivity

Gene symbol	Alterations in DIRC	Sensitivity of		Drugs	Association with chemosensitivity (cancer, drug)	Reference no.
		UCs	DCs			
TP53		S, R*	R, S	CDDP, DOX	Yes (brain)	223–229
					Yes (NCI-panel)	230
					No (breast, DOX)	231
					No (breast, DOX, PTX)	232
					No (lung, PTX)	22
MDM2		S, R	S	CDDP, DOX, PTX		169, 233–238
TP73			R	CDDP, ETP		239, 240
BCL2	U, D	R		CDDP, CPT, DOX	Yes (breast, DOX)	164, 198, 231, 241–244
					Yes (lung, PTX)	22
					No (breast, DOX)	232
BCL2L1	NC	R	S	CDDP, PTX		243–251
MCL1			S	DTIC		252
BAX	NC	S	R	CDDP, ETP, 5-FU	No (breast, DOX)	231, 244, 253–260
					No (lung, PTX)	22
BIRC4		NC	S	PTX		261, 262
BIRC5		R	S	CDDP, ETP		263–265
TNFRSF6	NC		S	CDDP	Yes (lung, DOX)	10, 242
CASP3		S		CDDP, DOX, ETP	No (lung, DOX)	10, 266–268
CASP8			R	CDDP		261
HSPB1	C	R	S	DOX		52, 269–273

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): NC, no change; R, resistant; S, sensitive. Drugs: CDDP, cisplatin; CPT, irinotecan; DOX, doxorubicin; DTIC, dacarbazine; ETP, etoposide; PTX, paclitaxel; 5-FU, 5-fluorouracil
*Resistant in mutant *TP53* over-expressed cells

Table 8. Gene categories and association with in vitro chemosensitivity

Category	No. of genes	Total no. of studies	No. of studies showing association (%)
Transporter	15	13	7 (54)
Drug target	8	5	3 (69)
Target associated protein	7	0	0 (0)
Intracellular detoxifier	7	6	6 (100)
DNA repair	10	3	2 (67)
DNA damage recognition protein	2	0	0 (0)
Cell cycle	6	5	3 (60)
Mitogenic signal	5	3	1 (33)
Survival signal	2	0	0 (0)
Transcription factor	4	3	0 (0)
Cell adhesion-mediated drug resistance protein	1	0	0 (0)
Apoptosis	13	12	5 (42)
Total	80	50	22 (44)

but not selected in the current study, because they have never caught the scientific eye for some reasons. Thus, the results of this study may be significantly influenced by publication bias. Nonetheless, we do believe that these genes have been selected reasonably carefully, and that they may be helpful for establishing a clinical predictive chemosensitivity test.

While the association between alterations of the 80 genes and the chemosensitivity of various cell lines was evaluated in 50 studies, significant association was observed in only 22 (44%) (Table 8). The cellular functions of a gene vary among cell types and experimental conditions. The evaluation of the gene functions, however, was conducted under only limited cellular contexts in these studies, as expected. Thus, for example, the conditions of a gene transfection experiment may differ from those of an experiment to evaluate the chemosensitivity for many cell lines. The gene functions may not necessarily be examined under all possible conditions, but the evaluation must be conducted under conditions similar to those in the clinical setting in order to develop clinical chemosensitivity testing using these genes.

The other possibility for the poor correlation to *in vitro* chemosensitivity may be that more than one gene alterations are involved in the chemosensitivity of tumors. This may be discussed from the standpoint of the signal transduction pathway and from the cellular standpoint. From the standpoint of the signal transduction pathway, more than one gene may be involved in the reaction to a cytotoxic agent. One of the best examples is cooperation of *TP53* with another member of the p53 family, p73 (*TP73*), in the response to both DNA damage and chemosensitivity (3,4). From the cellular standpoint, several pathways may work additively, antagonistically, or complementally in determining the chemosensitivity of the cell. This can be understood well from the context of induction and inhibition of apoptosis being controlled by pro-apoptotic and anti-apoptotic pathways. Thus, it would be important to study several pathways at the same time, or to evaluate the net effect of the involvement of various pathways.

Complex factors influencing the cellular chemosensitivity may be operative on a tumor *in vivo*, in such a way that the tumor may exhibit highly heterogeneous gene alterations: that the tumor cells may interact with various host cells, including immune cells, fibroblasts and vascular endothelial cells; and that the differences in the distance between each tumor cell and blood vessels may affect the exposure level of tumor cells to a drug. No systematic approach has been developed to include this complex interplay of factors in the study of cellular chemosensitivity, although studies on cell adhesion-mediated drug resistance may be partly helpful.

Among the six genes for which the association was shown in two or more *in vitro* studies, four encode classical drug resistance proteins which are known to inhibit the drug target interaction. These proteins are relatively specific for the drug as well as the cell type; e.g. *TYMS* is critical for 5-fluorouracil sensitivity. Thus, *TYMS* is a good candidate for chemosensitivity testing in patients with colorectal cancer who are treated with 5-fluorouracil (Table 2). *MVP* is involved in the transport of doxorubicin, therefore, it would be of interest to examine the association between the expression of *MVP* and the drug response in patients with breast cancer; the association of *MVP* with chemosensitivity has been evaluated only for brain tumor and lung cancer cell lines, to date (Table 1). However, the remaining two of the six genes, *TP53* and *BCL2*, are associated with apoptosis, and therefore may be relatively cell-type specific. Since all the three *in vitro* studies using breast cancer cell lines failed to show any associations between alterations of these genes and the chemosensitivity, the association should be evaluated in other tumor types in the clinical setting (Table 7).

The recently developed cDNA microarray technique allows analysis of the mRNA expression of more than 20 000 genes at once, and as many as 100–400 genes have been statistically shown as potentail chemosensitivity-related genes in various studies (5–7). The 80 genes in the current study were selected theoretically based on their known functions, and their contribution to *in vitro* chemosensitivity was shown in the experiments. Thus, it would be of interest to evaluate the expression profiles of these genes by cDNA microarray analysis, even if the difference in expression between sensitive and resistant cell lines does not reach statistical significance.

In conclusion, 80 *in vitro* chemosensitivity associated genes were identified from a review of the literature, which

may be considered to be future candidates for clinical predictive chemosensitivity testing.

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Conflict of interest statement

None declared.

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ORIGINAL ARTICLE

Genetic linkage of *UGT1A7* and *UGT1A9* polymorphisms to *UGT1A1*6* is associated with reduced activity for SN-38 in Japanese patients with cancer

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Abstract
Purpose The phenotypic effects of *UGT1A7* and *UGT1A9* genetic polymorphisms on the in vivo pharmacokinetics of irinotecan were examined.
Methods Eighty-four Japanese patients with cancer who received irinotecan-based chemotherapy were enrolled. Polymorphisms present in *UGT1A7* (*T* to *G* transversion at −57 and *UGT1A7*2* to **9*), *UGT1A9* (9 or 10 repeat of *T* at −118 [−118(*T*)9 or 10] and *UGT1A9*2* to **5*), and *UGT1A1* (*UGT1A1*6*, *UGT1A1*27*, and *UGT1A1*28*) were analyzed for all patients. Pharmacokinetics of irinotecan were examined in 52 patients.
Results The most frequent haplotype (haplotype I, 56.7%, 95% CI 53.1–60.4) consisted of polymorphisms related to normal catalytic or transcriptional activity [*T* at −57 and **1* of *UGT1A7*, −118(*T*)10 of *UGT1A9*, and *UGT1A1*1*]. The second most frequent haplotype (haplotype II, 15.0%, 95% CI 12.4–18.3) consisted of polymorphisms related to reduced catalytic or transcriptional activity [−57*T* > *G* and **3* of *UGT1A7* and −118(*T*)9 of *UGT1A9* linked to *UGT1A1*6*]. The $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratios in three patients homozygous for haplotype II were significantly higher than those in 20 patients with I/I diplotype ($P = 0.011$). Neither of these patients had *UGT1A1*28*.
Conclusion Genetic linkage of *UGT1A7* and *UGT1A9* polymorphisms to *UGT1A1*6*, related to reduced catalytic and transcriptional activities of UGTs, is associated with the decreased glucuronosyltransferase activity for SN-38 in Japanese patients with cancer.

Keywords Irinotecan · SN-38 · Polymorphism · *UGT1A7* · *UGT1A9* · *UGT1A1*6*

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Introduction

Irinotecan is a camptothecin analogue with high-antitumor efficacy that acts by inhibiting topoisomerase I. Irinotecan is a prodrug metabolized to its active metabolite SN-38, which is further conjugated by hepatic UDP-glucuronosyltransferase (UGT) 1A1, to yield the more polar, inactive SN-38 glucuronide (SN-38G) [1]. A (TA)7 within the promoter of the human *UGT1A1* gene (*UGT1A1*28*) has been associated with reduced glucuronidation capacity as well as with irinotecan-related dose-limiting toxicity, most commonly diarrhea and neutropenia [2–4].

Although UGT1A7 and UGT1A9 also participate in the glucuronidation of SN-38 in vitro [5–7], the in vivo roles of these UGTs remain poorly understood as compared with that of UGT1A1. UGT1A7 and UGT1A9, as well as UGT1A1 are encoded by a single *UGT1A* gene located on chromosome 2q37. UGT1A7 is expressed exclusively in the oropharynx, esophagus, stomach, and pancreas [8–12], but is absent in the liver [13]. In contrast, UGT1A9 is expressed in the liver, kidney, small intestine, colon, and reproductive organs such as the testis and ovary [8–10].

Functionally significant genetic polymorphisms have been described for *UGT1A7* and *UGT1A9* [6, 14, 15]. *UGT1A7*3*, **4*, **5*, **8*, and **9* and *UGT1A9*3* and **5*

reduce catalytic activity for SN-38 [6, 14, 15]. Polymorphisms affecting transcriptional activity have also been identified [16, 17]. Lankisch et al. [16] have shown that a *T* to *G* transversion at −57 (−57*T* > *G*), located in the putative TATA box of the *UGT1A7* gene, is related to a reduction in promoter activity to 30%. The 9 repeat of *T* allele at −118 [−118(*T*)9], resulting in lower transcriptional activity than −118(*T*)10, has been found in the 5′-flanking region of the *UGT1A9* gene [17]. Clinically, Carlini et al. [18] have shown that *UGT1A7*3/*3* is significantly associated with a good antitumor response to irinotecan and lack of severe gastrointestinal toxicity in patients with metastatic colorectal cancer. Furthermore, they have proposed that homozygosity for the presence of −118(*T*)9 sites in the *UGT1A9* gene is significantly related to enhanced response and reduced toxicity. These results have suggested that *UGT* genotypes causing low catalytic or transcriptional activity are associated with better responses to irinotecan. This hypothesis is consistent with the notion that low-catalytic activities or expression levels of UGTs might increase plasma concentrations of SN-38, enhancing the clinical response to irinotecan. However, the increased plasma concentrations of SN-38 seen in patients with *UGT* gene polymorphisms seem unlikely to relate to the reduced toxicity.

To gain better insight into the in vivo roles of UGT1A7 and UGT1A9 in SN-38 glucuronidation, we studied the relation between genotypes of *UGT1A7* and *UGT1A9* and the pharmacokinetics of irinotecan. First, we examined genetic polymorphisms present in *UGT1A7*, *UGT1A9*, and *UGT1A1* in 84 Japanese patients with cancer who received irinotecan-based chemotherapy. We then studied the relation between the genotype and the pharmacokinetics of irinotecan in 52 of these patients.

Patients and methods

Materials

Irinotecan, SN-38, and SN-38G were kindly supplied by Yakult Honsha (Tokyo, Japan). All chemicals and solvents were of the highest grade commercially available.

Patients

The study group comprised 84 Japanese patients (males/females, 52/32) with cancer (50 colons, 18 stomachs, seven ovaries, seven lungs, and two others) who received irinotecan monotherapy or various regimens of irinotecan-based combined chemotherapy from November 2004 through June 2006. A subset of the patients in the present study was included in the previous study [19]. The median age of the patients was 62 years (35–85). All patients gave informed consent in writing for their peripheral blood samples and medical information to be used for research. The study protocol was approved by the Institutional Review Board of Saitama Medical School.

Treatments

For monotherapy, irinotecan was given weekly at a dose of 100 mg/m^2 for the first 3 weeks of a 4-week cycle, or every 2 weeks at a dose of 150 mg/m^2. In combination with fluorouracil and leucovorin (IFL regimen), 100 mg/m^2 of irinotecan was administered weekly for the first 4 weeks of a 6-week cycle. In the FOLFIRI regimen, irinotecan was administered at 2-week intervals at doses of 150 or 180 mg/m^2. In combination with cisplatin (IP regimen), irinotecan was given at a dose of 50–70 mg/m^2 on day 1 of a 4-week cycle and at the same dose on day 15. For each of these regimens, irinotecan (50–180 mg/m^2) was infused over the course of 90 min.

Genotyping

Genomic DNA was extracted from 200 µl of peripheral blood, which had been stored at −80°C until analysis, with the use of a QIAamp Blood Kit (QIAGEN GmbH, Hilden, Germany).

UGT1A1

Two polymorphisms (G71R [**6*] and P229Q [**27*]) were analyzed by the polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP) described by Ando et al. [2]. The TATA box polymorphism (TA)7 (**28*) was determined by the direct sequencing method described by Ando et al. [2].

UGT1A7

The *UGT1A7* promoter sequence was amplified by PCR, and sequence analysis was performed to determine −57*T* > *G* as described by Lankisch et al. [16], with minor modifications. Briefly, the reaction mixture consisted of 2.5 mM MgCl2 and 1.25 u of AmpliTaq Gold polymerase in a final volume of 50 µl.

Genotype of exon 1 was determined by direct sequencing of a PCR product that spans all of the polymorphic sites (N129K and R131K [**2*], N129K, R131K and W208R [**3*], W208R [**4*], G115S [**5*], E139D [**6*], N129K, R131K and E139D [**7*], N129K, R131K, E139D, and W108R [**8*] and G115S, N129K, and R131K [**9*]) as described by Carlini et al. [18], with minor modifications. Briefly, the reaction mixture consisted of 2.5 mM MgCl2

and 1.25 u of AmpliTaq Gold polymerase in a final volume of 50 μl.

UGT1A9

Five polymorphisms (−118(*T*)9 or 10 [based on assigning the A in the translation start codon as +1] [18], C3Y [*2], M33T [*3], Y242X [*4], and D256N [*5]) were evaluated by direct DNA sequencing of a PCR amplicon spanning all of the polymorphic sites as described by Carlini et al. [18], with minor modifications. Briefly, the reaction mixture consisted of 2.5 mM MgCl2 and 1.25 u of AmpliTaq Gold polymerase in a final volume of 50 μl.

Statistical analysis

Allele and genotype frequencies for each polymorphic allele in the *UGT1A7*, *UGT1A9*, and *UGT1A1* genes were determined by using SNPAlyze 5.0 (Dynacom, Yokohama, Japan). The significance of deviations from Hardy–Weinberg equilibrium was tested with the program SNPAlyze 5.0. Linkage disequilibrium analysis to make pairwise two-dimensional map of correlation coefficient r^2 and Lewontin's coefficient *D'* among single nucleotide polymorphisms, haplotype and diplotype configurations (combinations of haplotypes) analyses were also performed by an expectation-maximization-based algorithm using SNPAlyze 5.0. Polymorphisms not in Hardy–Weinberg equilibrium were excluded from the haplotype analysis.

The statistical significance of differences in the ratio of the AUC for SN-38 to that for SN-38G ($AUC_{SN\text{-}38}$/$AUC_{SN\text{-}38G}$) was assessed with the Mann–Whitney *U*-test. This and other statistical analyses were performed with SPSS for Windows, version 12.0J (SPSS Japan Inc., Tokyo Japan). Differences were considered statistically significant when the two-tailed *P*-value was less than 0.05.

Pharmacokinetic analysis

Pharmacokinetic analysis was performed in 52 subjects of this study. Blood samples for pharmacokinetic analysis were obtained during the first cycle of treatment. If necessary, blood samples were obtained during subsequent cycles of treatment to analyze pharmacokinetics. The blood samples were taken from the arm opposite the infusion site at the beginning of irinotecan infusion and 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h after the end of the infusion. The samples were immediately centrifuged, and the plasma was stored at −80°C until analysis.

Total (lactone and carboxylate) plasma concentrations of irinotecan, SN-38, and SN-38G were analyzed by reverse-phase high-performance liquid chromatography (HPLC) as described by Araki et al. [19]. The lower limit of quantification for irinotecan was 5 ng/ml (7.4 nM), and those for SN-38 and SN-38G were 0.5 ng/ml (1.2 and 0.88 nM). The intraassay and interassay coefficients of variation for irinotecan and the metabolites were less than 10%.

The area under the time-versus-concentration curve (AUC, μM h) from the beginning of the infusion to the time of obtaining the last blood sample was calculated by the linear trapezoidal rule, using a computer program (WinNonlin version 4.01 software, Pharsight Corporation, Mountain View, Calif).

Results

Polymorphisms in *UGT1A7*, *UGT1A9*, and *UGT1A1* in Japanese patients with cancer

The genotypes of *UGT1A7*, *UGT1A9*, and *UGT1A1* were determined in the 84 patients. The allele frequencies of the *−57T > G* allele of *UGT1A7* and the −118(*T*)9 allele of *UGT1A9*, associated with reduced transcriptional activity, were 25.0 and 38.1%, respectively. The allele frequency of these polymorphisms in Japanese was reported to be 22, and 34–40%, respectively [17, 20]. The frequency of *UGT1A7*3*, related to the reduced catalytic activity, and *UGT1A7*2* were 29.2 and 11.9%, respectively. Previous studies demonstrated that the frequencies of *UGT1A7*3* and *UGT1A7*2* in Japanese were about 25 and 15%, respectively [21, 22]. The allele frequencies of *UGT1A1*6* and *UGT1A1*28* were 22.6 and 9.5%, respectively. The reported frequencies were 15.1 and 13.3%, respectively [23]. All allele frequencies, except for those of *UGT1A1*27* (0.6%) and *UGT1A9*5* (0.6%), were in Hardy–Weinberg equilibrium ($P > 0.05$). There was no patient harboring *UGT1A7*4–*9* and *UGT1A9*2–*4*.

Diplotypes or genotypes of *UGT1A7*, *UGT1A9*, and *UGT1A1* found in the patients are shown in Table 1. The frequencies of homozygosity for *−57T > G* of *UGT1A7* and of *UGT1A7*3/*3* were both 7.1%, and that of homozygosity for −118(*T*)9 of *UGT1A9* was 10.7%. The frequencies of *UGT1A1*6/*6* and *UGT1A1*6/*28* were both 4.8%.

Linkage disequilibrium analysis

The result of the linkage disequilibrium analysis is shown in Fig. 1. We found that −118(*T*)9 of *UGT1A9* was highly linked with *UGT1A7* variants to cause N129K and R131K ($r^2 = 0.88$, $D' = 1$). *−57T > G* of *UGT1A7* gene was linked with *UGT1A7*3* ($r^2 = 0.7$, $D' = 0.93$). *UGT1A1*6* was linked with *UGT1A7*3* ($r^2 = 0.55$, $D' = 0.88$) and *−57T > G* of *UGT1A7* ($r^2 = 0.48$, $D' = 1$). r^2 and *D'*-values seen between *UGT1A1*6* and −118(*T*)9 of *UGT1A9* were 0.29 and 0.79, respectively.

Table 1 Diplotypes or genotypes of the *UGT1A7*, *UGT1A9*, and *UGT1A1* genes found in Japanese patients with cancer

Gene	Genotype or diplotype	Number	Frequency (%)
UGT1A7	*−57 T/T*	48	57.1
	−57 T/G	30	35.8
	−57 G/G	6	7.1
	**1/*1*	26	31.0
	**1/*2*	14	16.7
	**2/*2*	1	1.2
	**1/*3*	33	39.2
	**2/*3*	4	4.8
	**3/*3*	6	7.1
UGT1A9	−118(*T*) 9/9	9	10.7
	−118(*T*) 9/10	46	54.8
	−118(*T*) 10/10	29	34.5
	**1/*1*	83	98.8
	**1/*5*	1	1.2
UGT1A1	**1/*1*[a]	38	45.1
	**1/*28*	12[b]	14.3
	**6/*28*	4	4.8
	**1/*6*	26	31.0
	**6/*6*	4	4.8

The number of patients was 84

[a] *UGT1A1*1* was defined as the allele not possessing **28*, **6*, and **27*

[b] The **28* and **27* were assumed to exist on the same allele [2, 22]

Haplotype structures of *UGT1A1*, *UGT1A7*, and *UGT1A9*

Since *UGT1A1*27* and *UGT1A9*5* were not in Hardy–Weinberg equilibrium ($P < 0.05$), these loci were excluded from haplotype analysis. Sai et al. [22] have found that *UGT1A1*6* and *UGT1A1*28* do not exist on the same allele. Therefore, we performed haplotype analysis with the *UGT1A1*1*, *UGT1A1*6*, and *UGT1A1*28* alleles.

Fifteen haplotypes estimated are shown in Table 2. The most frequent haplotype (haplotype I, 56.7%, 95% CI 53.1–60.4) consisted of polymorphisms related to normal catalytic or transcriptional activity. The second most frequent haplotype (haplotype II, 15.0%, 95% CI 12.4–18.3) consisted of polymorphisms related to reduced catalytic or transcriptional activity (*−57T > G* and **3* of *UGT1A7*, −118(*T*)9 of *UGT1A9*, and *UGT1A1*6*). In addition to the linkage disequilibrium analysis, these findings supported the genetic linkage among *UGT1A7* and *UGT1A9* polymorphisms and *UGT1A1*6*.

Relations between reduced glucuronidation capacity for SN-38 and haplotype structures of *UGT1A1*, *UGT1A7*, and *UGT1A9*

Pharmacokinetic analysis of irinotecan and its metabolites SN-38 and SN-38G was performed in 52 subjects. Their characteristics are summarized in Table 3. The distribution of $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratios is shown in Fig. 2. The median of the $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratios was 0.5. Diplotype configurations in the *UGT1A7*, *UGT1A9*, and *UGT1A1* genes were estimated by the haplotype analysis with the all of 84 Japanese patients with cancer. The diplotype configurations for 52 patients with pharmacokinetic data are shown in Table 4. The number of patient(s) showing $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratio(s) higher than 1.0 and the ratio(s) are also described in the Table 4. Three patients were homozygous for haplotype II. The $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratios observed in these patients were 1.40, 1.10, and 1.11, respectively. The patient showing the 1.40 of $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$

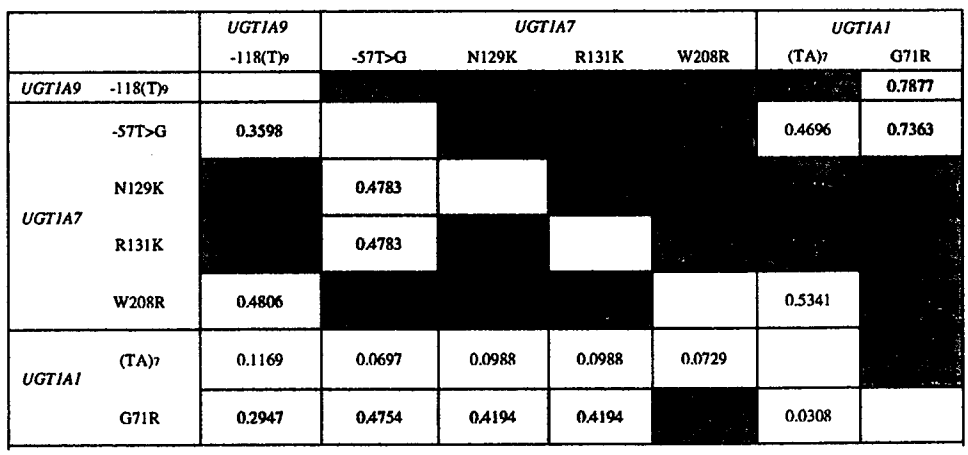

Fig. 1 Linkage disequilibrium analysis for *UGT1A7*, *UGT1A9* and *UGT1A1* single nucleotide polymorphisms r^2 (*lower red*) and *D'* (*upper blue*) values are shown in each *square*. r^2-values; *open squares*, 0–0.25; *pink squares*, 0.25–0.5; *red squares*, 0.5–1.0: *D'*-values; *open squares*, 0–0.6; *light blue squares*, 0.6–0.8; *dark blue squares*, 0.8–1.0. *UGT1A7*2* causes N129K and R131K. *UGT1A7*3* causes N129K, R131K, and W208R

Table 2 Haplotype structures of the *UGT1A7*, *UGT1A9*, and *UGT1A1* genes in Japanese patients with cancer

Haplotype	*UGT1A7*		*UGT1A9*	*UGT1A1*	Frequency (%) (95%CI)
	−57	Exon1	−118(*T*)9 or 10		
I	*T*	**1*	10	**1*	56.7 (53.0–60.4)
II	*G*	**3*	9	**6*	15.0 (12.4–18.3)
III	*T*	**2*	9	**1*	8.64 (6.67–10.8)
IV	*G*	**3*	9	**28*	5.05 (3.31–6.73)
V	*T*	**3*	9	**6*	3.31 (2.04–4.80)
VI	*T*	**1*	10	**28*	2.18 (1.09–3.59)
VII	*G*	**3*	10	**6*	1.87 (0.827–2.93)
VIII	*G*	**3*	9	**1*	1.85 (0.924–3.05)
IX	*T*	**2*	9	**28*	1.45 (0.563–2.58)
X	*T*	**3*	9	**28*	0.834 (0.338–1.75)
XI	*G*	**2*	9	**6*	0.684 (0.318–1.46)
XII	*T*	**3*	9	**1*	0.624 (0.306–1.27)
XIII	*T*	**2*	9	**6*	0.613 (0.302–1.24)
XIV	*T*	**3*	10	**6*	0.595 (0.298–1.19)
XV	*G*	**2*	10	**6*	0.510 (0.108–1.16)

*UGT1A1*27* and *UGT1A9*5* were not included in this analysis because these loci were not in Hardy–Weinberg equilibrium

Table 3 Demographic characteristics of patients participating in the pharmacokinetic study

		Number
Age (year)[a]	62 (42–85)	52
Sex	Male	32
	Female	20
Performance status	0	33
	1	17
	2	2
Creatinine (mg/dl)[a]	0.66 (0.42–1.15)	52
Total bilirubin (mg/dl)[a]	0.5 (0.2–1.1)	52
Tumor type	Colon	27
	Stomach	14
	Ovarian	4
	Others	7
Type and dose of irinotecan therapy (mg/m^2)[a]	Monotherapy 100 (50–150)	12
	IFL 100 (50–150)	3
	FOLFIRI 180 (150–180)	22
	IP 80 (60–100)	15
Toxicity	Grade 4 neutropenia	5
	Grade 3 neutropenia	4
	Grade 3 diarrhea	0

[a] The values are expressed as the median with the range in parentheses

received the same dose of irinotecan in the second cycle of treatment (FOLFIRI, 180 mg/m^2) as the first cycle. The $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratio during the second cycle was also high (2.73). The relation between the $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratios and diplotype configurations of I/I, I/II, and II/II was examined (Fig. 3). The Kruskal–Wallis test for these three data sets (three diplotypes) yielded *P*-value of 0.027. The $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratios in the three patients who were homozygous for the haplotype II were significantly higher than those in 20 patients with I/I diplotype ($P = 0.011$). The diplotype configurations II/II occurred at frequency of 3.6% in Japanese patients with cancer (Table 4).

Discussion

Our study showed that genetic linkage of *UGT1A7* and *UGT1A9* polymorphisms to *UGT1A1*6*, related to low catalytic and transcriptional activities of UGTs, was associated with the pharmacokinetics of irinotecan and with reduced glucuronosyltransferase activity for SN-38. Given that UGT1A7 and UGT1A9 might be involved in the glucuronidation of SN-38 in vivo, the lower glucuronidation capacity for SN-38 in patients homozygous for haplotype II was probably caused not only by *UGT1A1*6*, but also by polymorphisms in the *UGT1A7* and *UGT1A9* gene.

Previous studies have shown that the gene product of *UGT1A7*3* has about 50% lower catalytic activity for SN-38 than that of *UGT1A7*1* [6, 14]. The *−57T > G* polymorphism in the putative TATA box of *UGT1A7* reduces promoter activity to 30% [16]. However, an in vivo role of UGT1A7 might be less likely, because this enzyme is not expressed in liver or intestine [8–13]. Yamanaka et al. [17] have demonstrated that −118(*T*)9 polymorphism is related to the 2.6-fold lower transcriptional activity of the *UGT1A9*

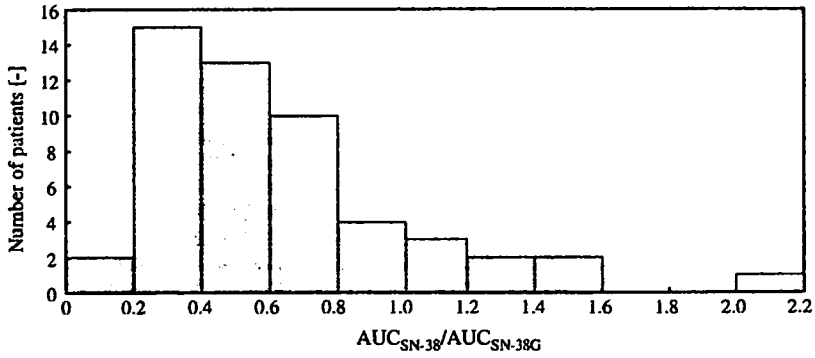

Fig. 2 Distribution of $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratios. The median value of the $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratios was 0.5

Table 4 Diplotype configurations in *UGT1A7*, *UGT1A9*, and *UGT1A1* in Japanese patients with cancer

Haplotype[a]/ haplotype	All patients		Fifty-two patients with pharmacokinetic data
	n	Frequency (%)	*n*
I/I	25	29.7	20 (1; 1.39)[b]
I/II	14	16.6	11 (2; 1.16 and 1.24)
I/III	10	11.8	6
I/IV	6	7.1	1
I/V	4	4.8	1
I/VI	1	1.2	
I/VII	2	2.4	1
I/VIII	2	2.4	2
I/IX	3	3.6	1
I/X	1	1.2	
I/XII	1	1.2	
I/XIII	1	1.2	1
I/XIV	1	1.2	
II/II	3	3.6	3 (3; 1.40, 1.10, and 1.11)
II/III	2	2.4	2
II/VI	2	2.4	1 (1; 1.04)
II/XV	1	1.2	
III/V	1	1.2	
III/XI	1	1.2	1 (1; 1.42)
IV/V	1	1.2	
IV/VII	1	1.2	1 (1; 2.16)
IV/VIII	1	1.2	
Total	84		52

[a] The haplotype numbers are identical to those shown in Table 2

[b] Numbers in parenthesis represent the number of patient(s) showing $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratio(s) higher than 1.0 and the ratio(s)

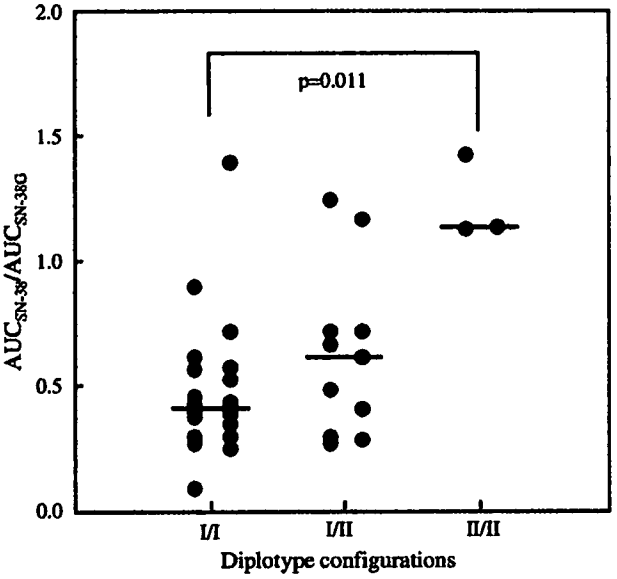

Fig. 3 Relationship between plots of the AUC for SN-38 versus the ratio of the AUC for SN-38 and the diplotype configurations. Numbers of patients for respective diplotype configurations I/I, I/II, and II/II were 20, 11, and 3. *Lines indicate* median-value

gene. On the other hand, the no association between −118(*T*)9 polymorphism and UGT1A9 protein level has been reported [24]. Innocenti et al. [25] have demonstrated that SN-38 glucuronidation rate was higher in patients heterozygous for −118(*T*)9 than in patients with homozygous −118(*T*)10. Taking these results into account, an alternative hypothesis is rose.

(1) UGT1A1 has a role in SN-38 glucuronidation in vivo, whereas UGT1A7 and UGT1A9 do not.
(2) The polymorphisms in the *UGT1A7* and *UGT1A9* gene are linked to *UGT1A1*6* because of the close proximity of these loci (<100 kb) (GeneBank, AF297093).
(3) The reduced glucuronidation capacity for SN-38 in patients homozygous for haplotype II is principally caused by *UGT1A1*6*.

If patients homozygous for polymorphism in *UGT1A7* and *UGT1A9* together with *UGT1A1*1* (e.g., haplotype VIII) could be identified, it would theoretically be possible to evaluate the roles of UGT1A7 and UGT1A9 by comparing the in vivo pharmacokinetics of irinotecan in these patients

with the pharmacokinetics in patients homozygous for haplotype II. However, patients homozygous for haplotype VIII were not found in the present study. Therefore, from the results of the present study, it is difficult to assess the in vivo roles of UGT1A7 and UGT1A9 in SN-38 glucuronidation on the basis of the phenotypic effects of haplotype II. Further studies should need.

In whites, the allele frequencies of $-57T > G$ and *3 in the *UGT1A7* gene and of $-118(T)9$ in the *UGT1A9* gene are higher than those in Japanese (39, 36, and 61%, respectively) [16, 17, 23, 25, 26]. The frequency of *UGT1A1*6* in whites is estimated to be lower than that in Asians, including Japanese [2, 25, 27], whereas the frequency of the *UGT1A1*28* allele is higher in whites than in Asians [2, 28]. These findings suggest that the genetic linkages of these polymorphisms in *UGT1A7* and *UGT1A9* to *UGT1A1*6*, frequently seen in Japanese patients with cancer, probably occur at lower frequencies in whites.

In a patient homozygous for haplotype II showing the $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratio of 1.40, grade 3 neutropenia developed during the second cycle of treatment with irinotecan, given in the same dose as the first cycle (FOLFIRI, 180 mg/m^2). The other patient with the diplotype II/II with the $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratio of 1.11 suffered from grade 4 neutropenia 10 days after the first cycle of irinotecan treatment (monotherapy, 100 mg/m^2). Therefore, the third cycle of irinotecan treatment for the patient was discontinued. These results might indicate that the configuration of diplotype (II/II) might be important for predicting not only lower SN-38 glucuronidation capacity, but also the risk of irinotecan-related toxicity in Japanese patients with cancer.

The diplotype configuration in a patient showing the highest $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratio (2.16) consisted of haplotype VII and haplotype IV (Table 4). The structure of haplotype VII was nearly consistent with that of haplotype II, except for -118(*T*)10 of *UGT1A9*, indicating genetic linkages of $-57T > G$ and *3 of *UGT1A7* to *UGT1A1*6*. The polymorphisms of *UGT1A7* and *UGT1A9* in haplotype IV were similar to those seen in haplotype II and were linked to *UGT1A1*28*. The higher $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratio seen in the patient may be related to this diplotype configuration. Furthermore, the patient suffered from the grade 4 neutropenia at the first treatment of irinotecan (150 mg/m^2) with FOLFIRI regimen. Therefore, the dose of irinotecan was reduced to 100 mg/m^2 at the second treatment. Although the $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ seen in the patient at the second treatment was still high (1.56), the severe toxicity was not observed.

Among nine patients showing $AUC_{SN\text{-}38}/AUC_{SN\text{-}38G}$ ratios higher than 1.0, 6 patients did not suffer from irinotecan-related sever toxicity. According to our data, dose of irinotecan was not necessarily correlated to the sever toxicity in these of nine patients. Factors determining the sensitivity to the irinotecan-related toxicity need to be examined.

Recently, the novel *UGT1A9* intronic *c.* 855 + 399*C* > *T* polymorphism has been reported to appear as a predictor of SN-38 glucuronidation levels in the liver [29]. The most of the liver samples used in this study were from Caucasian, and did not include those from Japanese. Therefore, it is of interest to know the impact of this polymorphism on the irinotecan pharmacokinetics in Japanese patients with cancer.

In genotype-pharmacokinetic association analysis, Han et al. [30] had recently reported that *UGT1A1*6/*6* ($n = 6$), *UGT1A7*3/*3* ($n = 6$), and $-118(T)9/9$ in the *UGT1A9* ($n = 11$) were associated with significantly lower glucuronosyltransferase activity in Korean patients, despite they did not examine the effects of $-57T > G$ variant in *UGT1A7* gene. The results obtained by Han et al. and by us indicated that the combination of genotypes of *UGT1A7*, *UGT1A9* and *UGT1A1* might be important to predict atypical irinotecan pharmacokinetics and reduced glucuronosyltransferase activity in Asian patients with cancer.

In conclusion, our study showed that genetic linkages of *UGT1A7* and *UGT1A9* polymorphisms to *UGT1A1*6*, related to low catalytic and transcriptional activities of UGTs, is associated with the pharmacokinetics of irinotecan and reduced glucuronosyltransferase activity for SN-38.

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