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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
CCNND1	–	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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Irinotecan pharmacokinetics/pharmacodynamics and *UGT1A* genetic polymorphisms in Japanese: roles of *UGT1A1*6* and **28*

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Objectives SN-38, an active metabolite of irinotecan, is detoxified by glucuronidation with UGT1A isoforms, 1A1, 1A7, 1A9, and 1A10. The pharmacogenetic information on *UGT1A* haplotypes covering all these isoforms is important for the individualized therapy of irinotecan. Associations between *UGT1A* haplotypes and pharmacokinetics/pharmacodynamics of irinotecan were investigated to identify pharmacogenetic markers.

Methods Associations between *UGT1A* haplotypes and the area under concentration curve ratio (SN-38 glucuronide/SN-38) or toxicities were analyzed in 177 Japanese cancer patients treated with irinotecan as a single agent or in combination chemotherapy. For association analysis, diplotypes of *UGT1A* gene segments [(*1A1, 1A7, 1A9, 1A10*), and Block C (common exons 2–5)] and combinatorial haplotypes (*1A9-1A7-1A1*) were used. The relationship between diplotypes and toxicities was investigated in 55 patients treated with irinotecan as a single agent.

Results Among diplotypes of *UGT1A* genes, patients with the haplotypes harboring *UGT1A1*6* or **28* had significantly reduced area under concentration curve ratios, with the effects of *UGT1A1*6* or **28* being of a similar scale. A gene dose effect on the area under concentration curve ratio was observed for the number of haplotypes containing **28* or **6* (5.55, 3.62, and 2.07 for 0, 1, and 2 haplotypes, respectively, $P<0.0001$). In multivariate analysis, the homozygotes and double heterozygotes of **6* and **28* (**6/*6, *28/*28* and **6/*28*) were significantly associated with severe neutropenia in 53 patients who received irinotecan monotherapy.

Conclusions The haplotypes significantly associated with reduced area under concentration curve ratios and neutropenia contained *UGT1A1*6* or **28*, and both of them should be genotyped before irinotecan is given to Japanese and probably other Asian patients. *Pharmacogenetics and Genomics* 17:497–504 © 2007 Lippincott Williams & Wilkins.

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Keywords: diplotypes, genetic polymorphism, haplotype, irinotecan, SN-38, UGT1A1

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Introduction

Irinotecan, an anticancer prodrug, is widely applied for colorectal, lung, stomach, ovarian, and other various cancers. It is activated by carboxylesterases to SN-38 (7-ethyl-10-hydroxycamptothecin), which shows antitumor activity by inhibiting topoisomerase I [1,2]. SN-38 is subsequently glucuronidated by uridine diphosphate glucuronosyltransferases (*UGTs*) to form an inactive metabolite, SN-38 glucuronide (SN-38G) [3]. Dose-limiting toxicities of irinotecan are diarrhea and leukopenia [4], and reduced activity for SN-38G formation is closely related to severe toxicities [5]. Among UGT isoforms, UGT1A1 is abundant in both the liver and intestine and is thought to be mainly responsible for inactivation of SN-38 [3,6]. Genetic polymorphisms of *UGT1A1* result in reduced enzyme activity and increased toxicity by irinotecan. A significant association of *UGT 1A1*28*, a repeat polymorphism of the TATA box (-40_-39insTA) [3,7], with severe irinotecan-induced diarrhea/leukopenia was first reported in a retrospective study of Japanese cancer patients [8]. Subsequent pharmacogenetic studies in Caucasians have shown close associations of **28* with reduced glucuronidation of SN-38 and/or severe neutropenia/diarrhea [9–12]. These

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studies have clearly indicated that *28 is a good genetic marker for individualized irinotecan therapy. On the basis of these observations, the Food and Drug Administration of the United States has approved an amendment of the label for Camptosar (irinotecan HCl) and added a warning to consider a reduction in the starting dose of irinotecan for *28 homozygous patients (NDA 20-571/S-024/S-027/S-028).

There is significant racial difference in *UGT1A1* polymorphisms among Asians, Caucasians, and Africans [13]. Although the association of *UGT 1A1*28* with toxicities by irinotecan was first described in Japanese patients, its frequency in Japanese is one-third of that in Caucasians. Another low-activity allele *6 [211G > A(G71R)], which is not detected in Caucasians or Africans, is as frequent as the *28 allele in Japanese. Moreover, the area under concentration curve (AUC) ratio of SN-38G to SN-38 was decreased in patients having *6 haplotypes [14].

In addition to UGT1A1, recent studies have suggested possible contributions to SN-38G formation by UGT1A7, 1A9, and 1A10 [15–17], which are expressed in the gastrointestinal tract, the liver and intestine, and extrahepatic tissues, respectively [18]. Altered activity resulted from genetic polymorphisms of these isoforms, including *1A7*3* [387T > G(N129K), 391C > A(R131K), 622T > C(W208R)], *1A9*22* (-126_-118$T_9 > T_{10}$), *1A9*5* [766G > A(D256N)], and *UGT1A10*3* [605C > T(T202I)], but clinical relevance of these polymorphisms is yet to be elucidated [16,19–24]. Moreover, close linkages among *1A9*, *1A7*, and *1A1* polymorphisms were found in Caucasians and Asians in an ethnic-specific manner [20,25–27]. Therefore, comprehensive investigation that covers these genes, along with linkages among the polymorphisms, is needed, in each ethnic population, to evaluate associations between the genetic polymorphisms and pharmacokinetics, as well as clinical outcomes of irinotecan therapy.

Recently, we have analyzed the segmental and block haplotypes of *1A8*, *1A10*, *1A9*, *1A7*, *1A6*, *1A4*, *1A3* and *1A1*, and the common exons 2–5 (Block C) in a Japanese population, including the 177 cancer patients treated with irinotecan, and showed close linkages between the haplotypes, that is, *1A9*22* and *1A7*1*, *1A7*3* and *1A1*6*, and *1A7*3* and *1A1*28* [28]. Preliminary results of *UGT1A1* pharmacogenetics on 85 of these cancer patients were reported previously [14]. In the current study, we investigated the pharmacogenetics of irinotecan, focusing on diplotypes of the *UGT1A* complex covering *1A1*, *1A7*, *1A9*, *1A10*, and Block C (exons 2–5) of 177 patients, so as to elucidate haplotypes or genetic markers associated with altered glucuronidation of SN-38 and toxicities.

Methods

Patients and treatment schedule

Patients with cancers who started chemotherapy with irinotecan at two National Cancer Center Hospitals (Tokyo and Kashiwa, Japan) were eligible if they had not received irinotecan previously. Other eligibility criteria included bilirubin ≤ 2 mg/dl, aspartate aminotransferase (GOT) ≤ 105 IU/l, alanine aminotransferase (GPT) ≤ 120 IU/l, creatinine ≤ 1.5 mg/dl, white blood cell count $\geq 3000/\mu l$, performance status of 0–2, and at least 4 weeks after the last chemotherapy (2 weeks for radiotherapy). Exclusion criteria were diarrhea, active infection, intestinal paralysis or obstruction, and interstitial pneumonitis. The ethics committees of the National Cancer Center and the National Institute of Health Sciences approved this study, and written informed consent was obtained from all participants.

Irinotecan was administered as a single agent or in combination chemotherapy at the discretion of attending physicians. Doses and schedules were according to approved usage in Japan; intravenous 90-min infusion at a dose of 100 mg/m^2 weekly or 150 mg/m^2 biweekly. In terms of combination chemotherapy, the dose of irinotecan was reduced according to clinical protocols.

Genetic polymorphisms of UGT1As and pharmacokinetics

Detailed assay methods for genotypes of the *UGT1A* gene complex were reported previously [14,28]. In this study, we focused on the genetic variations in *UGT1A1*, *1A7*, *1A9*, and *1A10* and common exons 2–5, as they have been reported to contribute to the SN-38 glucuronidation. Haplotype analysis covering these regions was performed in our previous study [28], and haplotypes of each *UGT1A* segment [exon 1 for *1A1*, *1A7*, *1A9*, or *1A10*; and Block C (common exons 2–5)] are summarized in Fig. 1.

Pharmacokinetic analysis for irinotecan was performed as described previously [14]. Briefly, heparinized blood was collected before administration of irinotecan, as well as 0 and 20 min, and 1, 2, 4, 8, and 24 h after termination of the first infusion of irinotecan. Plasma concentrations of irinotecan, SN-38 and SN-38G were determined by the high-performance liquid chromatography [29], and AUC was calculated by the trapezoidal method using WinNonlin version 4.01 (Pharsight Corporation, Mountain View, California, USA). Associations between genotypes and the AUC ratio (AUC of SN-38G/AUC of SN-38) were evaluated in 176 patients.

Monitoring and toxicities

A complete medical history and data on physical examinations were recorded before the irinotecan therapy. Complete blood cell counts with differentials and platelet counts, as well as blood chemistry, were measured once a week during the first 2 months of irinotecan treatment. Toxicities were graded according to the Common Toxicity Criteria of National Cancer Institute version 2. Association of genetic factors with irinotecan toxicities was analyzed primarily in patients who received irinotecan as a single agent.

Fig. 1

UGT1A1

Region		Enhancer	Promoter	Exon 1		Frequency
Nucleotide change		-3270 T>G	-40_-39 insTA	211 G>A	686 C>A	
Amino acid change				G71R	P229Q	
Marker allele		*60	*28	*6	*27	
Haplotype	*1					0.548
	*6					0.167
	*60					0.147
*28	*28b					0.138
	*28c					
	*28d					

UGT1A10

Region	Exon 1				Frequency
Nucleotide change	4 G>A	177 G>A	200 A>G	605 C>T	
Amino acid change	A2T	M59I	E67G	T202I	
Marker allele	*2T	*2	*67G	*3	
Haplotype *1					0.981
*2					0.006
*2T					0.003
*3					0.010
*67G					0.000

UGT1A7

Region	Exon 1				Frequency
Nucleotide change	387 T>G	391 C>A	392 G>A	622 T>C	
Amino acid change	N129K	R131K		W208R	
Marker allele	*2,*3	*2,*3	*2,*3	*3,*4	
Haplotype *1					0.630
*2					0.147
*3					0.223

Block C

Region		Exon.4	Exon.5		3'-UTR			Frequency
Nucleotide change		1091 C>T	1456 T>G	1598 A>C	*211(1813) C>T	*339 (1941) C>G	*440(2042) C>G	
Amino acid change		P364L	Y486D	H533P				
Marker allele		*364L	*7	*533P	*IB	*IB	*IB	
Haplotypes	*IA							0.864
*IB	*1b–*1j							0.127
	*533P							
	*7							0.003
	*364L							0.006

UGT1A9

Region	Promoter		Exon1			Frequency
Nucleotide change	-126_-118 T9>T10	-126_-118 T9>T11	422 C>G	726 T>G	766 G>A	
Amino acid change			S141C	Y242X	D256N	
Marker allele	*22	*T11	*141C	*4	*5	
Haplotype *1						0.347
*22						0.644
*141C						0.000
*4						0.000
*5						0.006
*T11						0.003

Haplotypes of *UGT1A* gene segments (*UGT1A1, 1A7, 1A9, 1A10*, and Block C) in 177 Japanese cancer patients. The tagging variations and haplotypes are shown. Variant alleles are indicated in grey. Definition of Block C haplotypes in our previous paper ([14]) (corresponding to Block 2) were slightly modified.

Statistical analysis

Statistical analysis on the differences in the AUC ratios (SN-38G/SN-38) among *UGT1A* genotypes was performed using the Kruskal–Wallis test, followed by nonparametric Dunnett's multiple comparison test, or with Wilcoxon test. Analysis of a gene–dose effect of each haplotype was performed using the Jonckheere–Terpestra test in the SAS system, version 5.0 (SAS Institute, Cary, North Carolina, USA). Relationship of *UGT1A* genetic polymorphisms to the toxicities of irinotecan was assessed by the χ^2 test via the use of using Prism version 4.0 (GraphPad Prism Software, San Diego, California, USA). The *P*-value of 0.05 (two-tailed) was set as a significant level, and the multiplicity adjustment was conducted for pharmacokinetics data with the false discovery rate [30].

To identify factors associated with the log-transformed AUC ratio of SN-38G/SN-38, multiple regression analysis was performed using age, sex, body surface area, dosage of irinotecan, history of smoking or drinking, performance status, coadministered drugs, serum biochemistry parameters at baseline, and *1A9-1A7-1A1* and Block C haplotypes (five or more chromosome numbers) or '*1A1*6* or *28'. For multiple regression analysis of neutropenia, variables included the absolute neutrophil count at baseline and the dosing interval, in addition to

the other patient background factors described above. The multivariate analyses were performed by using JMP version 6.0.0 software (SAS Institute). The variables in the final models for both AUC ratio and neutropenia were chosen by forward and backward stepwise procedures at significance levels of 0.25 and 0.05, respectively.

Results

Patients and UGT1A haplotypes

Patient demographics and information on the treatment are summarized in Table 1. In addition to UGT1A1, UGT1A7, 1A9, and 1A10 were also reported to glucuronidate SN-38 [15–17]. In our previous study, haplotype analysis covering the *1A9* to *1A1* (5′–3′) gene segments was conducted, and the combinatorial diplotypes (*1A9-1A7-1A1*) of the patients were determined. It must be noted that close linkages between *1A9*22* and *1A7*1*, between *1A7*2* and *1A1*60*, and between *1A7*3* and *1A1*6* or *1A1*28* were observed as described previously [28]. To clarify the linkages between these segmental haplotypes (*1A9*, *1A7*, and *1A1*), we grouped the combinatorial (*1A9-1A7-1A1*) haplotypes into four categories (A–D) based on the *1A1* haplotypes (**1*, **6*, **60*, and **28*). Each group was further divided into the subgroups based on the previously defined Block 9/6 (including *1A9*, *1A7*, and *1A6*) haplotypes (Table 2). The frequency of Group B haplotypes (*B1-B4*) harboring *1A1*6* was 0.167 and higher than that of Group D haplotypes (*D1-D6*) with **28* (0.138) in this population.

Association of 1A9-1A7-1A1 diplotypes to SN-38G formation

When relationship between the *UGT1A* diplotypes (*1A9-1A7-1A1*) and the SN-38G/SN-38 AUC ratio was analyzed in the 176 cancer patients the AUC ratio for the diplotypes of *B2/B2*, *D2/A1*, and *D1/B2* was statistically significantly lower than the *A1/A1* diplotype (Fig. 2). These diplotypes harbored *1A1*6*, **28* or both. Significant gene–dose effects of *B2* (among *A1/A1*, *B2/A1*, and *B2/B2*) and *C3* (among *A1/A1*, *C3/A1*, and *C3/C3*) were also observed (Fig. 2). As no significant differences in AUC ratios were observed between *D1/A1* and *D2/A1*, *D1/C3* and *D2/C3*, and *D1/B2* and *D2/B2*, the haplotype combination *1A9*1-1A7*3* or *1A9*22-1A7*1* was not influential on the AUC ratio.

As the effect of diplotypes harboring *UGT1A1* polymorphism was prominent, we grouped the whole gene (*1A9-1A7-1A1*) diplotypes according to the *1A1* diplotypes (the upper part of Fig. 2). Patients with **6* or **28* (except for **28/*28*) haplotypes had significantly lower AUC ratios than the wild-type (**1/*1*), and significant gene–dose effects were observed for **28* (among **1/*1*, **28/*1*, and **28/*28*) and **6* (among **1/*1*, **6/*1* and **6/*6*). A significant additive effect of **6* and **28* on the decreased AUC ratio was also observed when the values for **28/*1* were compared with those for **28/*6* (Fig. 2 and Table 3).

Regarding other polymorphisms, a statistically nonsignificant tendency to decrease the AUC ratio was observed for **60*

Table 1 Characteristics of Japanese cancer patients in this study

		No. of participants	
Age			
Mean/range	60.5/26–78	177	
Sex			
Male/female		135/42	
Performance status	0/1/2	84/89/4	
Combination therapy and tumor type (initial dose of irinotecan; mg/m^2)			
Irinotecan monotherapy	Lung (100)	21	
	Colon (150)	28	
	Others (100)	7	
With platinum-containing drug[a]	Lung (60)	58[b]	48 [60][c]
	Stomach (70)	9	9 [80][c]
	Others (60)	5	5 [80][c]
With 5-fluorouracil (including tegafur)	Colon (100 or 150)	34	
	Others (90 or 100)	2	
With mitomycin-C	Stomach (150)	10	
	Colon (150)	1	
With amrubicin	Lung (60)	2	
Previous treatment			
Surgery	Yes/no	85/92	
Chemotherapy	Yes/no	97/80	
Radiotherapy	Yes/no	26/151	
Smoking history	Yes/no	29/148	

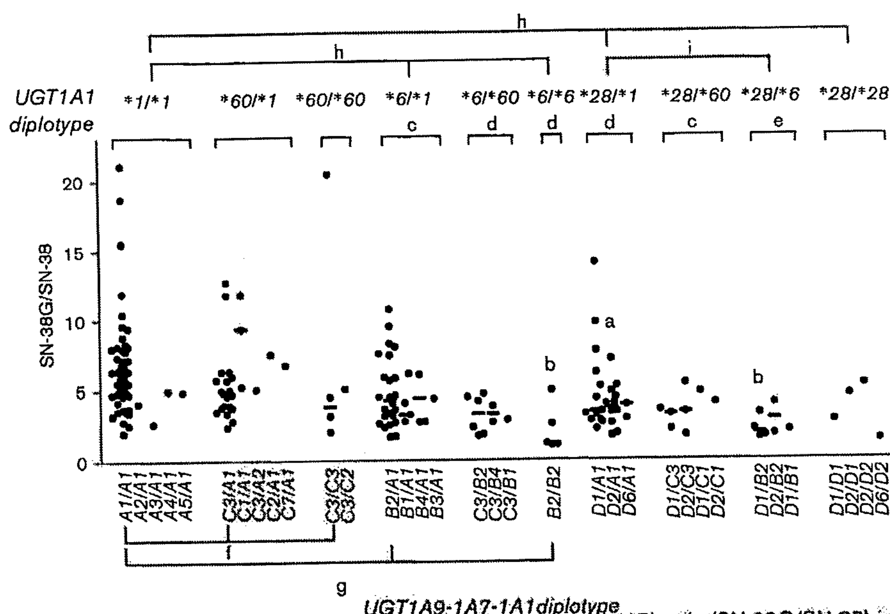
[a]Cisplatin, cisplatin plus etoposide or carboplatta.
[b]Two and eight patients received cisplatin and etoposide and carboplatin, respectively.
[c]Number of cisplatin-administered patients [initial dose of cinlatin (mg/m^2) is shown in brackets].

Table 2 Combinatorial haplotypes covering *UGT1A9*, *UGT1A7*, and *UGT1A1*

	Block haplotype[a]			Combination of segmental haplotypes	Cancer patients	
Haplotype	Block 9/6	Block 4	Block 3/1	1A9-1A7-1A1	*N*[b]	Frequency
A1[c]	*I	*1	*I	*22–*1–*1	189	0.534
	*I	*3	*I			
A3	*III	*1	*I	*1–*2–*1	2	0.006
A2	*II	*1	*I	*1–*3–*1	1	0.003
A4	*IV	*1	*I	*22–*3–*1	1	0.003
A5				*T11–*1–*1	1	0.003
B2[c]	*II	*1	*III			
	*II	*1	*VI	*1–*3–*6	47	0.133
	*II	*4	*VI			
B4	*IV	*1	*III	*22–*3–*6	6	0.017
B1	*I	*1	*III	*22–*1–*6	5	0.014
	*I	*1	*VI			
B3	*III	*1	*III	*1–*2- *6	1	0.003
C3[c]	*III	*3	*IV			
	*III	*1	*IV			
	*III	*3	*V	*1–*2–*60	44	0.124
	*III	*1	*V			
C1	*I	*3	*IV	*22–*1–*60	5	0.014
	*I	*1	*IV			
C2	*II	*3	*IV	*1–*3–*60	2	0.006
C7	*VII	*3	*V	*22–*2–*60	1	0.003
D1	*I	*1	*IIa	*22–*1–*28	23	0.065
	*I	*1	*IIc			
D2	*II	*1	*IIa			
	*II	*3	*IIa	*1–*3–*28	22	0.062
	*II	*1	*IIc			
D6	*VI	*1	*IIb	*1–*2–*28	4	0.011
				Total	354	1.000

[a]Block haplotypes described in Ref. [28] are shown for reference. *1A9* and *1A7* are included in block 9/6 and *1A1* is included in block 3/1.
[b]Number of chromosomes.
[c]Major combinatrial haplotypes.

Fig. 2



The association of *UGT1A* diplotypes with the reduced area under concentration curve (AUC) ratio (SN-38G/SN-38) in 176 Japanese cancer patients who received irinotecan. The whole gene (*1A9-1A7-1A1*) diplotypes are shown below the abscissa and the *UGT1A1* diplotypes are indicated in the upper part of the figure. Each point represents a patient value, and the median is indicated by a bar. Significant reductions in the AUC ratio were detected in the *B2/B2*, *D2/A1*, and *D1/B2* compared with *A1/A1* for the whole gene diplotypes [Kruskal–Wallis test (P=0.0009) followed by Dunnett's multiple comparison test]. As for the *1A1* diplotypes, significant reductions were detected in the **6/*1*, **6/*60*, **6/*6*, **28/*1*, **28/*60*, and **28/*6* compared with the **1/*1* group [Kruskal–Wallis test ($P<0.0001$) followed by Dunnett's multiple comparison test]. Gene–dose effects on the reduced AUC ratio were significant for **6* and **28* (Jonckheere–Terpestra test). A significant additive effect of **6* on the reduced AUC ratio by **28* was detected by comparing **28/*1* and **28/*6*. [a]$P<0.05$ and [b]$P<0.01$ against *A1/A1* group (Dunnett's multiple comparison test); [c]$P<0.05$, [d]$P<0.01$, and [e]P<0.001 against the **1/*1* group (Dunnett's multiple comparison test); [f]$P<0.05$, [g]$P<0.001$, and [h]$P<0.0001$ (Jonckheere–Terpestra test for gene–dose effect); [i]$P<0.01$ (Wilcoxon test).

($P = 0.1134$). No significant effects on the AUC ratio were observed for Block C (exon 2–5) haplotypes or rare variations including *1A10* (**2T*, **2*, or **3*) and *1A9* (**5*, **T11*).

Multiple regression analysis of the area under concentration curve ratio

We further assessed the impact of *UGT1A* genetic factors on the AUC ratio by multiple regression analysis. First, we used the *1A9-1A7-1A1* and Block C haplotypes as genetic factors. The AUC ratio was significantly associated with the haplotypes *B2*, *D1*, and *D2* and serum biochemistry parameters indicating hepatic or renal function before treatment. The Groups B and D haplotypes harbor *1A1*6* and **28*, respectively. The dependency on specific *1A7* or *1A9* polymorphisms, however, was not obtained, considering the contributions of both *D1* and *D2*. As *1A1*6* and **28* are mutually exclusive and their effects are comparable, we grouped *1A1*6* and **28* into the same category in the final multiple regression model (Table 4). The final model confirmed the significant contribution of this genetic marker (**6* or **28*) to the AUC ratio.

Effects of the genetic marker '*6 or *28' on pharmaco kinetic parameters

Then, a dose effect of the genetic marker '**6* or **28*' on pharmacokinetic parameters was further analyzed (Fig. 3). Patients with one haplotype harboring either **6* or **28* (**6/*1*, **6/*60*, **28/*1*, and **28/*60*) had lower SN-38G/SN-38 AUC ratios (median, 3.62; interquartile range, 2.74–5.18) than patients without **6* or **28* (**1/*1*, **60/*1*, and **60/*60*) (5.55, 4.13–7.26), and patients with two haplotypes harboring **6* or **28* (**6/*6*, **28/*28*, and **28/*6*) had the lowest AUC ratio (2.07, 1.45–3.62) ($P < 0.0001$, Fig. 3a). Similarly, the number of the **6* or **28*-containing haplotypes affected the AUC ratios of SN-38 to irinotecan (Fig. 3b). When the correlations

Table 3 AUC ratio of SN-38 glucuronide to SN-38 for *UGTIAI* diplotypes

Diplotype	Number of patients	AUC ratio		P-value[a] (vs. *1/*1)
		Median	Interquartile range	
*1/*1	55	6.13	4.72–7.79	
*1/*60	25	5.04	3.85–6.52	0.9803
*60/*60	5	4.48	2.57–12.74	0.8141
*6/*1	32	4.03	2.74–5.97	0.0126
*6/*60	9	2.84	2.09–4.33	0.0021
*6/*6	5	1.19	1.06–3.74	0.0012
*28/*1	26	3.65	2.76–5.21	0.0040
*28/*60	8	3.44	2.68–4.40	0.0261
*28/*6	7	2.03	1.65–3.26	<0.0001
*28/*28	4	3.65	2.05–4.92	0.2322

AUC, area under concentration curve.
[a]Dunnett's multiple comparison test.

between irinotecan dosage and the AUC of SN-38 were tested, different correlations were obtained according to the number of the haplotypes (Fig. 3c). The slope of regression line for one and two haplotypes harboring **6* or **28* was 1.4-fold and 2.4-fold greater, respectively, than that for the diplotype without **6* or **28*.

Associations of UGT1A1 genetic polymorphisms with toxicities

Association between genetic polymorphisms and toxicities was investigated in patients receiving irinotecan as a single agent. One patient was referred to another hospital 3 days after the first administration of irinotecan without evaluating toxicities and was lost in terms of follow-up. Therefore, association between genetic polymorphisms and toxicities was investigated in 55 patients. Six (11%) and 14 (25%) patients experienced grade 3 or greater diarrhea and neutropenia, respectively. As for the *1A9-1A7-1A1* diplotypes, a higher incidence of grade 3 or greater neutropenia was observed in *D1/B2* (*1A1*28/*6*) (100%, $n = 3$) than in *A1/A1* (11.8%, $n = 17$) ($P = 0.0088$, Fisher's exact test), indicating clinical impact of the genetic marker *1A1*6* or **28*. As for the dose effect of '**6 or *28*', incidences of grade 3 or 4 neutropenia were 14, 24, and 80% for 0, 1, and 2 haplotypes harboring these markers, respectively (Table 5). A significant association between '**6* or **28*'and neutropenia was also observed for 62 patients who received irinotecan in combination with cisplatin (Table 5). No association, however, was observed between diarrhea and the marker '**6* or **28*'.

Multivariate analysis for irinotecan toxicities

We further evaluated the effect of the genetic marker '**6* or **28*' on neutropenia in multivariate analysis, and confirmed a significant correlation of '**6* or **28*' with the nadir of absolute neutrophil counts (Table 6). Elevated alkaline phosphatase levels and the absolute neutrophil count at baseline were also significant.

Discussion

The association study with the *1A9-1A7-1A1* diplotypes revealed that the reduction in inactivation of SN-38, as well as neutropenia, was dependent on the Groups B and D haplotypes which corresponded to the *1A1*6* and **28* segmental haplotypes. Also, multivariate analyses clearly showed clinical significance of the genetic marker '**6* or **28*' for both pharmacokinetics and toxicity of irinotecan in Japanese patients (Tables 3 and 6). *UGT1A1*6* and **28* were mutually exclusive [14] and contributed to the reduction in glucuronidation of SN-38 to the same extent. Therefore, the activity of SN-38 glucuronidation in individuals depended on the number of the haplotypes harboring **6* or **28*. Although the role of *1A1*28* for irinotecan toxicity has been focused on [8–12], this study strongly suggests that **6* should be tested in addition to **28* before starting chemotherapy with irinotecan in Japanese patients.

The clinical importance of **6* for neutropenia by irinotecan was also supported by a recent report in Korean patients who received irinotecan and cisplatin [31]. Although no patients with irinotecan as a single agent were homozygous for **6* in our study, clinical significance of the double heterozygote, **6/*28*, was clearly demonstrated. Among patients treated with irinotecan in combination chemotherapy, the majority of patients received platinum agents in our study. A significant association of '**6* or **28*' with a higher incidence of grade 3 or 4 neutropenia was also observed in patients who received irinotecan and cisplatin (Table 5). These findings further support the necessity of testing '**6* or **28*' before irinotecan is given to patients.

As possible enhancement of toxicities by the **27* allele was suggested [8], we evaluated the effect of the **28c* haplotype, which had an additional single-nucleotide polymorphism [**27*; 686C > A(P229Q)] to the **28* allele (-40_-39insTA). In our cohort of patients, there were three **28c* heterozygotes (**28c/*1*) and one double heterozygote (**28b/*28c*). The values of the AUC ratio were within the range of variations of the **28* group, and no additional impact of **28c* was observed in relation to toxicities.

Although the decreasing trend of the AUC ratio for *1A1*60* (and combinatorial haplotype *C3*) was observed (Fig. 2), the contribution of *1A1*60* to toxicities was not clearly demonstrated in this study as reported in the Japanese retrospective study [32].

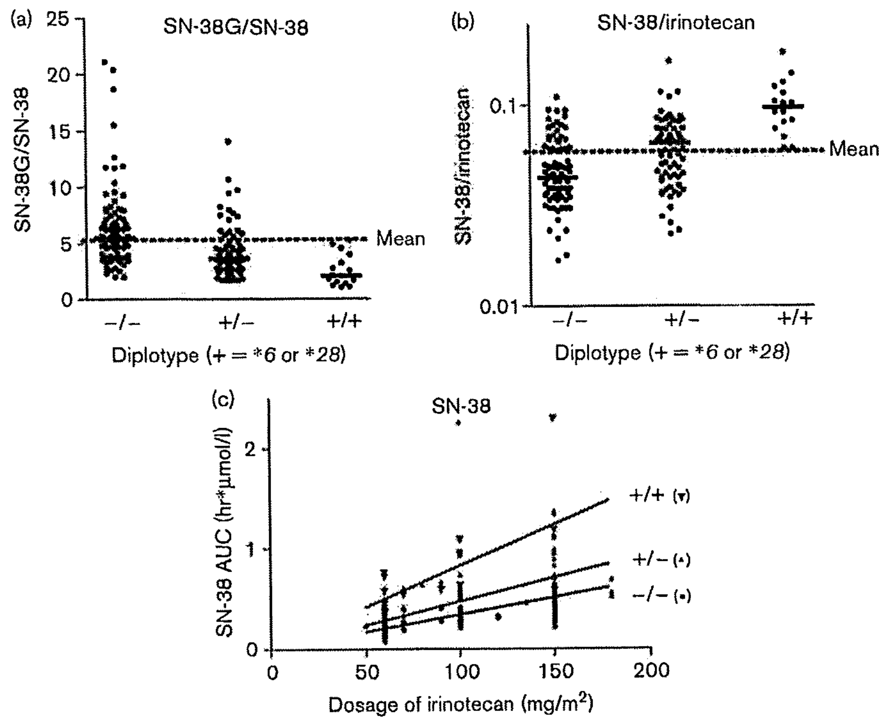
In addition to UGT1A1, recent studies have suggested possible contributions of UGT1A7, 1A9, and 1A10 to SN-38G formation [15–17]. An in-vitro study demonstrated that *1A7*3* [387T > G(N129K), 391C > A(R131K), 622T > C(W208R)] had reduced activity in terms of SN-38G formation [16]. Results of clinical studies, however, on the association between *1A7* polymorphisms and irinotecan toxicity/efficacy are inconsistent, whereas different populations with different combination therapies were used [19,20]. Furthermore, it was reported that the *UGT1A7* polymorphisms (**2* and **3*), which were linked to *1A9*1*, were associated with a lowered incidence

Table 4 Multiple regression analysis toward the AUC ratio (SN-38G/SN-38)[a]

Variable	Coefficient	F-value	*P*-value	R^2	Intercept	*N*
				0.410	0.8869	176
**6* or **28*	−0.189	70.2	<0.0001			
Age	0.005	8.88	0.0033			
Serum albumin level[b]	−0.136	9.92	0.0019			
Serum GOT and ALP[c]	0.070	8.88	0.0033			
Serum creatinine[d]	0.210	7.23	0.0079			

ALP, alkaline phosphatase; AUC, area under concentration curve.
[a]The values after logarithmic conversion were used as an objective variable.
[b]The absolute value (g/dl) before irinotecan treatment.
[c]Grade 1 or greater scores in both serum GOT and ALP before irinotecan treatment.
[d]Grade 1 or greater scores in serum creatinine before irinotecan treatment.

Fig. 3



Effects of the genetic marker of *UGT1A1* '*6 or *28' on the area under concentration curve (AUC) ratios of SN-38G/SN-38 (a) and SN-38/irinotecan (b), and SN-38 by irinotecan dosage (c) in 176 Japanese cancer patients after irinotecan treatment.

Table 5 Association of *UGT1A1*6* and **28* with irinotecan toxicities

Diplotype (+ = *6 or *28)	Number of patients	Diarrhea (grade 3)	Neutropenia (grade 3 or 4)
Irinotecan monotherapy			
−/−	21	3 (14.3%)[a]	3 (14.3%)
+/−	29	2 (6.90%)	7 (24.1%)
+/+	5	1 (20.0%)	4 (80.0%)
P-value[b]		0.8500	**0.0117**
P-value[c]		0.3889	**0.0124**
With cisplatin			
−/−	35	1 (2.9%)	20 (57.1%)
+/−	20	2 (10.0%)	14 (70.0%)
+/+	7	1 (14.3%)	7 (100%)
P-value[b]		0.1747	**0.0315**
P-value[c]		0.3886	0.0863

[a]Percentage of the patient number in each diplotype is indicated in parentheses.
[b]Chi-squared test for trend.
[c]Fisher's exact test, (−/− and +/−) vs. +/+.

Table 6 Multiple regression analysis of the nadir of absolute neutrophil counts in the patients with irinotecan monotherapy

Variable	Coefficient	F-value	*P*-value	R^2	Intercept	*N*
				0.3942	643	53
Serum ALP[a]	−349.9	12.2	0.0010			
Neutrophil count before irinotecan treatment	0.2466	13.5	0.0006			
*6 or *28	−369.1	6.40	0.0146			

[a]Grade 1 or greater scores of serum ALP before irinotecan treatment.

of diarrhea in the irinotecan/capecitabine regimen, in which diarrhea was a major toxicity [20]. A highly frequent allele *1A9*22* with an insertion of T into the nine T repeats in the promoter region (-126_-118$T_9 > T_{10}$) was shown to have an enhanced promoter activity in an in-vitro reporter assay [21], whereas 1A9 protein expression levels did not change in the clinical samples [22]. Rare variations, *1A9*5* [766G > A(D256N)] and *UGT1A10*3* [605C > T(T202I)], were shown to cause reduced activity *in vitro*, but their clinical importance is still unknown [23,24]. Moreover, close linkages among *1A9*, *1A7*, and *1A1* polymorphisms were found in Caucasians and Asians in an ethnic-specific manner [20,25–28].

Our study also revealed close linkages between *1A9*22* and *1A7*1*, *1A7*3* and *1A1*6* or **28* [28]. This fact makes it difficult to draw firm conclusions about the effects of *1A7*3* and *1A9*22* themselves. It is, however, reasonable to conclude that the degree of neutropenia depends on the activity of UGT1A1, because UGT1A1 is a major UGT1A enzyme in the liver and plays a primary role for regulating plasma concentrations of SN-38.

Taken together, for practical application to individualized irinotecan therapy, genotyping of *UGT1A1*6* and **28* would be beneficial and necessary in Japanese cancer patients to avoid severe adverse reactions. The frequency

of homozygotes for '*6 or *28' (namely, *6/*6, *6/*28, and *28/*28) is approximately 10%, which is comparable to the frequency of *28 homozygotes in Caucasian populations. In our study, it may be difficult to establish definite guidelines for dose reductions of irinotecan for patients homozygous for '*6 or *28'. Considering, however, 2.4-fold steep relationship between the dose of irinotecan and the AUC of SN-38 for patients homozygous for '*6 or *28' compared with patients without '*6 or *28' (Fig. 3c), the dose for patients homozygous for '*6 or *28' should be reduced to a half of the dosage recommended for other patients. Prospective studies are necessary to confirm the validity of the recommendation for dose reduction in Japanese cancer patients homozygous for '*6 or *28'.

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CYP2C8 haplotype structures and their influence on pharmacokinetics of paclitaxel in a Japanese population

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Objective CYP2C8 is known to metabolize various drugs including an anticancer drug paclitaxel. Although large interindividual differences in CYP2C8 enzymatic activity and several nonsynonymous variations were reported, neither haplotype structures nor their associations with pharmacokinetic parameters of paclitaxel were reported.

Methods Haplotype structures of the *CYP2C8* gene were inferred by an expectation-maximization based program using 40 genetic variations detected in 437 Japanese patients, which included cancer patients. Associations of the haplotypes and paclitaxel pharmacokinetic parameters were analyzed for 199 paclitaxel-administered cancer patients.

Results Relatively strong linkage disequilibriums were observed throughout the *CYP2C8* gene. We estimated 40 haplotypes without an amino-acid change and nine haplotypes with amino acid changes. The 40 haplotypes were classified into six groups based on network analysis. The patients with heterozygous **1G* group haplotypes harboring several intronic variations showed a 2.5-fold higher median area under concentration–time curve of C3′-*p*-hydroxy-paclitaxel and a 1.6-fold higher median value of C3′-*p*-hydroxy-paclitaxel/paclitaxel area under concentration–time curve ratio than patients bearing no **1G* group haplotypes ($P<0.001$ for both comparisons by Mann–Whitney *U*-test). No statistically significant differences, however, were observed between patients with and without the **1G* group (haplotypes) in clearance and area under concentration–time curve of paclitaxel, area under concentration–time curve of 6α-hydroxy-paclitaxel and 6α-, C3′-*p*-dihydroxy-paclitaxel, and area under concentration–time curve ratio of 6α-hydroxy-paclitaxel/paclitaxel.

Conclusion *CYP2C8*1G* group haplotypes were associated with increased area under concentration–time curve of C3′-*p*-hydroxy-paclitaxel and area under concentration–time curve ratio of C3′-*p*-hydroxy-paclitaxel/paclitaxel. Thus, **1G* group haplotypes might be associated with reduced CYP2C8 activity, possibly through its reduced protein levels. *Pharmacogenetics and Genomics* 17:461–471 © 2007 Lippincott Williams & Wilkins.

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Introduction

Cytochrome P450s (CYPs) catalyze oxidative metabolism of a wide variety of exogenous chemicals and endogenous compounds. Human CYP2C subfamily consists of four members, CYP2C18, CYP2C19, CYP2C9, and CYP2C8, all of which are located in tandem on chromosome 10q23–24 in the order listed above [1]. CYP2C8 is a clinically important enzyme, which metabolizes various drugs such as the anticancer drug paclitaxel (PTX), the antiarrhythmic drug amiodarone, the insulin secretagogue repaglinide, the HMG-CoA reductase inhibitor cerivastatin, and the nonsteroidal antiinflammatory drug ibuprofen [1]. This enzyme is also involved in the oxidation of retinoids and fatty acids including arachidonic acid [1].

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Up to 38-fold interindividual variability has been reported on PTX 6α-hydroxylation and rosiglitazone *p*-hydroxylation and *N*-desmethylation by CYP2C8 [2,3]. Effects of CYP2C8 genetic polymorphisms on metabolic activities have also been studied. Two polymorphisms first identified were 805A > T (Ile269Phe, *CYP2C8*2*) and 416G > A/1196A > G (Arg139Lys, Lys399Arg, *CYP2C8*3*). The **2* and **3* alleles were mainly found in Africans with 0.04–0.18 frequencies, and in Caucasians with 0.10–0.23 frequencies, respectively [1]. Both alleles were associated with decreased enzymatic activities for PTX 6α-hydroxylation *in vitro* [4–6]. *CYP2C8*4* allele (792C > G, Ile264Met) was found in British Caucasians [6]. We found 475delA (*CYP2C8*5*) in Japanese, and this allele leads to a frame shift at codon 159 followed by a stop codon at residue 177 [7]. We also found five additional polymorphisms (*CYP2C8*6* to **10*) in Japanese [8]. Among them, *CYP2C8*7* (556C > T, Arg186X) and **8* (556C > G, Arg186Gly) are different nucleotide variations at the same position. The former variation results in the stop codon, and the latter leads to an amino-acid substitution with a markedly reduced hydroxylation activity to PTX *in vitro*. Recently, two additional variations, *CYP2C8*13* (669T > G, Ile223Met) and **14* (712G > C, Ala238Pro), have been reported [9].

To date, a few reports have shown the impact of *CYP2C8*3* alleles on drug pharmacokinetics. The presence of **3* was associated with reduced clearance and increased area under concentration-time curve (AUC) of (*R*)-ibuprofen [10]. In contrast, significantly reduced AUC and C_{max} of repaglinide were observed in the patients with heterozygous **3* but not in patients with heterozygous **4* [11]. As for PTX, previous studies failed to show the influence of *CYP2C8* variations on PTX pharmacokinetics [12,13].

Haplotypes, linked polymorphisms on the same chromosome, often show more precise and strong association with phenotypes such as adverse reaction and/or pharmacokinetics of drugs than individual polymorphisms [14]. In this study, we determined/inferred haplotype structures of the *CYP2C8* gene using genetic polymorphisms detected in 437 Japanese patients. Then, association analysis was performed between the haplotypes and pharmacokinetic parameters for PTX and its metabolites. PTX is metabolized to form C3′-*p*-hydroxy-PTX (3′-*p*-OH-PTX) and 6α-hydroxy-PTX (6α-OH-PTX): both metabolites are further hydroxylated to 6α-,C3′-*p*-dihydroxy-PTX (diOH-PTX) [2,15,16]. CYP2C8 metabolizes PTX and 3′-*p*-OH-PTX into 6α-OH-PTX and diOH-PTX, respectively. Another enzyme, CYP3A4, metabolizes PTX and 6α-OH-PTX into 3′-*p*-OH-PTX and diOH-PTX, respectively. Previously, we showed that a *CYP3A4* haplotype affected the pharmacokinetics of these metabolites [9]. In this study, effects of *CYP2C8* haplotypes on PTX metabolism were investigated.

Materials and methods

Patients for DNA sequencing

A total of 437 Japanese patients (235 cancer patients administered PTX, 106 arrhythmic patients, and 96 epileptic patients) participated in this study. This population included 54 patients, who were previously used to identify the *CYP2C8*5* allele and four intronic variations [7], and seven patients with *CYP2C8*6* to **10* [8], **13* and **14* alleles [9]. Written informed consent was obtained from all participating patients. The ethical review boards of the National Cancer Center, the National Cardiovascular Center, the National Center of Neurology and Psychiatry, and the National Institute of Health Sciences approved this study.

Polymerase chain reaction conditions and DNA sequencing

Genomic DNA was extracted from whole blood leukocytes. First, the entire *CYP2C8* gene except for −8.8 and −1.9 kb enhancer regions was amplified in two portions (from the promoter region to exon 5, and exons 6–9) using the primer sets listed in the 'first polymerase chain reaction (PCR)' section of Table 1. Amplification was performed from 200 ng of genomic DNA using 1.25 units of Z-T (Takara Bio. Inc., Shiga, Japan) with 0.2 μmol/l of the primer sets. The first PCR conditions were 30 cycles of 98°C for 5 s, 55°C for 5 s, and 72°C for 190 s. Then, each exon (except for simultaneous amplification of exons 2 and 3) was amplified by Ex-Taq (1.25 units) with a set of primers (0.2 μmol/l) listed in the 'second PCR' section of Table 1 (primers were designed in the intronic regions or promoter region). The second-round PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. As for the −8.8 and −1.9 kb enhancer regions, amplification was performed directly from 50 ng of genomic DNA under the same conditions as in the second round PCR. Thereafter, the PCR products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, Ohio, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) with the primers listed in the 'Sequencing' section of Table 1. For the −8.8 and −1.9 kb enhancer regions, promoter region, exon 4, and exons 7–9, the primer sets for the second PCR were also utilized for sequencing. The excess dye was removed by a DyeEx 96 kit (Qiagen, Hilden, Germany). The eluates were applied to an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All detected variations were confirmed by repeating the PCR from the genomic DNA and sequencing of the newly generated PCR products. Genbank accession number NT_030059.12 was used for the reference sequence. Under conditions used, the −8.8 kb enhancer region (pregnanex receptor/constitutive androstane receptor-binding site and its surrounding region), −1.9 kb enhancer region (glucocorticoid receptor-binding site and

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Table 1 Primers used for the sequencing of *CYP2C8*

	Amplified and sequenced region	Forward primer		Reverse primer		Amplified length (bp)
		Sequences (5′–3′)	Position at 5′-end[b]	Sequences (5′–3′)	Position at 5′-end[b]	
First PCR	Promoter to exon 5	CTGTGGTGTAAGTGGTAATGAAC	15578696	AAAAGCCCTGAGAACCTATAATC	15563106	15591
	Exons 6–9	TAAGTATTTGTCCCAGTGCTCTC	15562092	TAGCAACTATACAAGCACGGG	15544271	17822
Second PCR	−8.8 kb	CCCAAAAAAAGAGCAGGTGTAGCCAT	15586590	TTACTGTCTGTCAAGTGGACCTATC	15586279	312
	−1.9 kb	CTGACCCACATTTTACTCAACTG	15579731	CCCAGTTTAGAGAGGAGAAAGTTAG	15579471	261
	Promoter	GTCCTGTTCTCCCAGAGTTTC	15578600	TCTCCAGAGTGAAAAGAGAAGC	15577623	978
	Exon 1	TCATAAATTCCCAACTGGTC	15578062	GAGCTTGCAGTGAGTGGAGA	15577279	784
	Exons 2–3	TGCTGAATGTGTTGAAGTGAGG	15576234	CTCCCTTGTCTCTGTGCTTC	15575334	901
	Exon 4	AGGCAGTGGATGTGAATAACC	15573481	TCTGTACCTAAAGATTGGAGGCTG	15572897	585
	Exon 5	TCTCAGCATACTATCACAAGGAC	15567211	TAAGGGCTATGTCAATGTGC	15566208	1004
	Exon 6	ACTAACCTAAGCAGCGAATGA	15554467	TTTTCATCTCCCCACCACAGCATT	15553696	772
	Exon 7	GGCTGGTTGTACTTCTGGAC	15551500	AATAGCAGAAAGTCCATCAAGC	15551034	467
	Exon 8	GAAGTGATGAAATAGAGCGGCAA	15547620	TAGTGGCAGAGTTCAGTCAAACC	15546922	699
	Exon 9	TGGGAATAAATAAAGAAATGACTG	15545899	GTCAGCATTAGAAAAGTATTAGCA	15545166	734
Sequencing[a]	Exon 1	CAGTGTTTCTCCATCATCACAGC	15577988	TTCAGAGGGAGTATTTTGCTTT	15577388	
	Exon 2	CATCACAGGCCATCTATAAGTGG	15576165	CCCCCTCACCCCAGTTACC	15575764	
	Exon 3	GGTAACTGGGGTGAGGGGG	15575782	CTCCCTTGTCTCTGTGCTTC	15575334	
	Exon 5	GGAACATTACACACTGGGGT	15567115	ATTATTTTTATTTCAAGAAGAGGG	15566396	
	Exon 6	ACTAACCTAAGCAGCGAATGA	15554467	TCTCTGTCATCCTCCTCCATT	15553904	

[a]Primer sets for the second PCR were used for the −8.8 kb, −1.9 kb, promoter, exons 4, 7, 8 and 9.
[b]The position in the reference sequence, NT_030059.12.

its surrounding region), promoter region (up to 890 bases upstream of the translational initiation site, including hepatocyte nuclear factor 4α-binding site) [17] and all nine exons and its flanking introns were successfully sequenced for all patients analyzed.

Linkage disequilibrium and haplotype analyses

Hardy–Weinberg equilibrium and linkage disequilibrium (LD) analyses were performed by SNPAlyze software (version 3.1, Dynacom Co., Yokohama, Japan), and a pairwise LD between variations was obtained for the $|D'|$ and rho square (r^2) values. Some haplotypes were unambiguously determined from patients with homozygous variations at all sites or a heterozygous variation at only one site. Separately, diplotypes (a combination of haplotypes) were inferred by LDSUPPORT software, which determines the posterior probability distribution of the diplotype configuration for each patient based on estimated haplotype frequencies [18]. Diplotypes of all patients were inferred with probabilities (certainties) of more than 0.95 except for 18 patients. Haplotypes without amino-acid changes were designated as **1*, and haplotypes with amino-acid changes were numbered according to the assignments by the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (*http://www.cypalleles.ki.se/cyp2c8.htm*). The estimated haplotypes (subtypes) were tentatively shown with numbers plus small alphabetical letters. The haplotypes (subtypes) already assigned by the Committee were described as numbers plus capital alphabetical letters (**1A*, **1B*, and **1C*). Network analysis was performed using haplotypes detected in more than two patients with Network 4.1.1.2 by median-joining algorithm (*http://fluxus-engineering.com/*) [19].

Patients administered PTX and pharmacokinetic analysis

Demographic data of 235 PTX-administered cancer patients including their eligibility criteria were described previously [9]. Of the 235 patients, 199 (185 nonsmall cell lung cancer, four thymic carcinoma, four breast cancer, and six other cancers) were treated with PTX at doses of 175–210 mg/m^2 (the high-dose group in the previous paper [9]) at the National Cancer Center, and used for analysis of associations between haplotypes and pharmacokinetic parameters. These patients consisted of 139 men and 60 women with a mean age of 60.8 (range: 29–81) years. All patients were naive to PTX and pretreated with dexamethasone and an antiallergic agent (diphenhydramine or chlorpheniramine maleate) as prophylactics against hypersensitivity reactions. Carboplatin or nedaplatin was coadministered to almost all patients immediately after PTX treatment. The ethical review boards of both the National Cancer Center and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all patients.

Methods for pharmacokinetic analysis were described previously, and the parameters obtained previously were used for the current association studies [9].

Statistical analysis for association studies

Differences in medians of pharmacokinetic parameters were analyzed by the Kruskal–Wallis test or the Mann–Whitney *U*-test. Statistical analysis was done using Prism v.4.00 (GraphPad Software Inc., San Diego, California, USA) and SAS v.8.2 (SAS Institute Inc., Cary,

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