表6　Grade 3 以上の有害事象

	FOLFOX		FOLFIRI	
	初回治療群 (n=26)	二次治療群 (n=17)	初回治療群 (n=10)	二次治療群 (n=5)
有害事象	5 (19.2)	12 (70.6)	6 (60.0)	2 (40.0)
減量投与	8 (30.8)	9 (52.9)	1 (10.0)	1 (20.0)
白血球減少	4	10	4	2
血小板減少	1			
意識障害		1		
食欲不振		1		
DIC			1	
脳梗塞			1	

(%)

表7　海外臨床試験におけるFOLFOX，FOLFIRI療法の成績

regimen	phase	RR (%)	PFS (m)	OS (m)	reference
FOLFOX4	Ⅲ	50	8.2	16.2	6)
FOLFOX4	Ⅲ (N9741)	45	8.7	19.5	17)
FOLFOX4	Ⅲ (OPTIMOX1)	59	9.0	19.3	18)
FOLFOX6	Ⅲ (C97)	54	8.0	20.6	5)
FOLFOX7	Ⅲ (OPTIMOX1)	59	8.7	21.2	18)
FOLFIRI	Ⅱ (3rd line)	6	18w	43w	19)
Douillard	Ⅲ	49	6.7	17.4	20)
FOLFIRI	Ⅲ (C97)	56	8.5	21.5	5)

その他2例）であった。FOLFOX療法，FOLFIRI療法ともに初回治療での施行により有効性，有害事象ともに二次治療より良好な結果が得られた。

考　　察

北信癌化学療法談話会は長野県の北部，および東部地区の医師，薬剤師，看護師による，主に消化器癌の化学療法の研鑽を目的とした会であり，年2回の研究講演会等を行っている。今回はFOLFOX，FOLFIRI療法を開始して約1年が経過することから，現状の分析，確認のために症例集積を行った。今回の集積の中では，初回治療でのFOLFOX療法の奏効率は50.0%であり，海外の臨床試験（表7）と同等の結果が実地臨床で得られた。

2004年のTournigandら[5]の報告によると，初回治療でのFOLFOX療法の奏効率は54%，FOLFIRI療法は56%であった。その中でTournigandらはdown stagingによって治癒切除に至った症例を解析しており，FOLFOX療法により治癒切除に至った症例は22%，その生存期間中央値は47カ月でさらに延長中とのことであった。今後，bevacizumab等の分子標的治

療薬の併用によりさらに成績は向上すると考えられ，化学療法の意義が生存期間の延長のみではなくなることも期待される。

その一方で，現在は施行可能で，かつ有効な化学療法をより円滑に実施することが重要と思われる。今回の集積では，FOLFOX症例においてGrade 3以上の末梢神経障害の発現はなかったが，これは投与回数中央値が6回（2～14回）と少なかったこと，Ca，Mg製剤の併用[3]が普及[21]していたことが影響していると考えられた。今後さらに長期間の経過観察を行い，Ca，Mg製剤の末梢神経障害抑制効果について検討を加えたい。他にFOLFOX療法を円滑に施行するためにすでに当会で実施している工夫として，5-FUのbolus投与を10分～15分程度かけて緩序に投与[21]することが挙げられる。また，FOLFOX療法の投与法は多くの場合mFOLFOX6 regimenが選択されていたが，その選択理由はインフューザーポンプを使用することにより2日目に来院する必要がなく，外来投与に移行しやすいことがある。さらに，嘔吐や白血球減少の一因とされる5-FUのbolus投与を1回に減らすことができ，5-FUの総投与量を増やせるという利点がある。

今回の集積はFOLFOX，FOLFIRI療法を開始してから約1年であり生存中の症例がほとんどのため，生存期間に関する解析はできなかった。今後の症例集積では有効性，有害事象の他に生存期間，治癒切除率について検討していく予定である。

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Biol. Pharm. Bull. 29(7) 1449—1453 (2006)

MDR1 T-129C Polymorphism can be Predictive of Differentiation, and Thereby Prognosis of Colorectal Adenocarcinomas in Japanese

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The expression level of MDR1 mRNA was evaluated in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues obtained from 21 Japanese patients. It was lower in the former than in the latter ($p=0.012$), suggesting its down-regulation as a consequence of malignant transformation of colorectal tissues, possibly with the suppression of differentiation. Relatively lower expression was suggested in moderately-differentiated colorectal adenocarcinomas than well-differentiated ones, but there was no statistical difference ($p=0.111$). MDR1 mRNA up-regulation was found in a colorectal adenocarcinoma cell line, HCT-15, after treatment with two typical differentiating agents, sodium butyrate and all-*trans* retinoic acid, suggesting its involvement in the cellular events, resulting in differentiation without malignant transformation. *MDR1* T-129C, but not G2677A,T and C3435T, was associated with the lower expression of MDR1 mRNA both in colorectal adenocarcinomas ($p=0.040$) and adjacent noncancerous colorectal tissues ($p=0.023$), possibly being an useful invasive marker predicting poorly-differentiated colorectal adenocarcinomas and thereby the poor prognosis of the patients, especially when no extra biopsy samples will be obtained. Further investigations with relatively large number of patients should be undertaken to confirm these preliminary results.

Key words colorectal adenocarcinoma; MDR1; expression; differentiation; genetic polymorphism

Multidrug resistant transporter MDR1/P-glycoprotein, the gene product of *MDR1*, is a glycosylated membrane protein of 170 kDa, belonging to the ATP-binding cassette superfamily of membrane transporters.[1—5] MDR1 was originally isolated from resistant tumor cells as part of the mechanism of multidrug resistance,[6—8] and a number of clinical investigations have suggested that the intrinsic or acquired overexpression of MDR1 in tumors resulted in a poor clinical outcome of cancer chemotherapy.[9] Moreover, over the last decade, it has been elucidated that human MDR1 is also expressed throughout the body to confer intrinsic resistance to the tissues by exporting unnecessary or toxic exogeneous substances or metabolites.[10] Various types of structurally unrelated drugs are substrates for MDR1, and MDR1 and other transporters are recognized as an important class of proteins for regulating pharmacokinetics and pharmacodynamics.[1—5] Furthermore, recent investigations have challenged the notion that MDR1 has evolved merely to facilitate the efflux of xenobiotics and have raised the possibility that MDR1 plays a fundamental role in regulating apoptosis.[11,12] Given the down-regulation of MDR1 expression during the differentiation of pluripotent stem cells along the myeloid lineage,[13] its potential implication in cell systems resulting in cell death or differentiation has been discussed for the last decade.

Numerous clinicopathological factors have been reported to have prognostic significance for colorectal cancer, including tumor invasion, nodal metastasis, differentiation and lymphocytic infiltration.[14] The importance of differentiation was already suggested in the 1920s, and the tumors have been graded into well-, moderately- and poorly-differentiated types. Most of colorectal cancers are assessed as well- or moderately-differentiated adenocarcinoma in the Japanese, being more frequently found than Caucasians, and the 5-year survival rate was reported to be 60—72%.[15,16] In contrast, it was only 32—46% for poorly-differentiated adenocarcinoma, although we rarely encountered this.[15,16] Thus, it is important to evaluate the differentiation grade accurately to decide on the patient management strategy; however, its usefulness is sometimes thought to be limited due to difficulties in the assessment and thereby reproducibility, encouraging us to search for alternative molecular markers,[17] or to establish a method of subclassification.[16]

In this study, the expression levels of MDR1 mRNA were assessed in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues obtained from 21 Japanese patients. Here, a relatively low expression level was suggested in moderately-differentiated adenocarcinomas than well-differentiated ones, but there were no patients with poorly-differentiated adenocarcinomas, and the study was not sufficiently powerful to reach statistical significance, and their relationship was attempted to be replicated in vitro using a colorectal adenocarcinoma cell line, HCT-15. The effects of genetic polymorphisms of the *MDR1* gene were also assessed, including T-129C, G2677A,T and C3435T, since they are re-

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ported to affect the expression of MDR1 and its mRNA,[1—5] and therefore possibly become novel and invasive markers predictive of prognosis.

MATERIALS AND METHODS

Human Colorectal Adenocarcinomas and Adjacent Noncancerous Colorectal Tissues Colorectal adenocarcinomas were obtained as surgical samples from 21 Japanese patients with primary colorectal adenocarcinoma diagnosed at Kobe University Hospital (10 men and 11 women). The average age was age 65.9±10.8 years (±S.D.; range, 29—79 years). Adjacent noncancerous colorectal tissues were simultaneously taken, and immediately after resection, these tissue samples were quickly stripped of connective tissue, snap-frozen and stored at −80 °C until processing. Informed consent was obtained from all subjects prior to their participation in the study. The protocol was approved by the Institutional Review Broad of Kobe University Hospital, Kobe University, Japan.

***MDR1* Genotyping** The colorectal adenocarcinomas and adjacent noncancerous colorectal tissues were cut up into small pieces in 1.5-ml microcentrifuge tubes, and then genomic DNA was extracted using a DNeasy Tissue Kit® (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. In this study, *MDR1* genotypes of C-145G (noncoding), T-129C (noncoding), G2677A,T (Ala893Thr, Ser) and C3435T (silent) were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as described previously,[18—21] and were confirmed by direct sequencing using an automatic ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The sequences of PCR primes for C-145G were 5′-TCA GCA TTC AGT CAA TCC GG-3′ (sense) and 5′-AGT AGC TCC CAG CTT TGC-3′ (anti-sense), and those for T-129C, G2677A,T and C3435T were described previously.[18—21] These primers were synthesized by Hokkaido System Science, Co., Ltd. (Sapporo, Japan).

MDR1 mRNA Levels in Colorectal Adenocarcinomas and Adjacent Noncancerous Colorectal Tissues Assessed by Real-Time Quantitative Reverse Transcription (RT)-PCR Total RNA was extracted from colorectal adenocarcinomas and adjacent noncancerous colorectal tissues using a RNeasy Mini kit (QIAGEN) and a RNase-Free DNase Set (QIAGEN) according to the manufacturers' protocols. The expression levels of MDR1 mRNA in were assessed by real-time quantitative RT-PCR analysis as described previously.[20—24] The sequences of primers for MDR1 were 5′-GCT CAG ACA GGA TGT GAG TTG GT-3′ (forward) and 5′-CCT GGA ACC TAT AGC CCC TTT AAC-3′ (reverse), and these primers were synthesized by Hokkaido System Science, Co., Ltd. The sequence of the TaqMan probe was 5′-AAA AAC ACC ACT GGA GCA TTG ACT ACC AGG-3′, and the probe was synthesized by Operon Biotechnologies, Inc. (Tokyo, Japan). The primers and probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an endogenous RNA control to normalize for differences in the amount of total RNA, were purchased from Applied Biosystems (TaqMan GAPDH Control Reagent Kit). In each run of the assay, the mRNA levels of GAPDH and MDR1 were analyzed in 4- or 5-fold serially diluted samples from an authentic human colon carcinoma cell line, Caco-2, and the assay was validated using a synthetic DNA template. The mRNA levels of MDR1 were expressed as a concentration relative to GAPDH mRNA.

Effect of Differentiating Agents on MDR1 mRNA Expression in a Colorectal Adenocarcinoma Cell Line, HCT-15 HCT-15 (passage 43) was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). HCT-15 cells were maintained in RPMI1640 culture medium (Invitrogen Corp., Carlsbad, CA, U.S.A.) supplemented with heat-inactivated 10% fetal bovine serum (FBS; CELLect® GOLD, MP Biomedicals, Irvine, CA, U.S.A.). The cells seeded at a density of 3.0×10^6 cells in 40 ml of culture medium in 175 cm^2 culture flasks (Nunclon™, Nalge Nunc International, NY, U.S.A.) were grown in an atmosphere of 95% air and 5% CO_2 at 37 °C, and subcultured every 3—4 d using a mixture of 0.02% EDTA and 0.05% trypsin (Invitrogen Corp.).

HCT-15 cells seeded at a density of 4×10^5 cells in 2 ml of culture medium in a 6-well plate (Nunclon™, Nalge Nunc International) were grown in an atmosphere of 95% air and 5% CO_2 at 37 °C. One day after, the culture medium was replaced, and an aqueous solution of sodium butyrate (NaB) or a dimethylsulfoxide (DMSO) solution of all-*trans* retinoic acid (ATRA), typical differentiating agents, was added to give the final concentrations of 0.1 or 1 mM for NaB and 1 or 10 μM for ATRA. The volume concentration of purified water or DMSO was less than 0.1%. After incubation for another 1 d at 37 °C, the cells were washed twice with ice-cold phosphate buffered saline, and then the cell pellets were prepared and stored at −80 °C until processing. The expression levels of MDR1 mRNA were evaluated as described above.

Statistical Analysis Values are given as the mean±standard deviation (S.D.). The statistical significance of differences between the mean values of MDR1 mRNA levels in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues were calculated using the Wilcoxon signed-rank test. The effects of differentiation or *MDR1* genotypes on the expression levels of MDR1 mRNA were analyzed by multiple comparisons with ANOVA followed by the Scheffé test, or by an unpaired Student's *t*-test. p values less than 0.05 were considered significant.

RESULTS

Figure 1 shows the relative concentrations of MDR1 mRNA in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues obtained from 21 Japanese patients. The expression levels were lower in colorectal adenocarcinomas (3.48±5.97, range 0.09—23.67) than adjacent noncancerous colorectal tissues (12.98±18.85, range 0.11—67.71, $p=0.012$ (Wilcoxon signed-rank test)) (Table 1). The ratio of expression in colorectal adenocarcinomas to adjacent noncancerous colorectal tissues was varied from 0.00 to 6.11 with an average of 0.87±1.50. Histological examination of colorectal adenocarcinomas showed that they consisted of well-differentiated ($n=5$), moderately-differentiated ones ($n=12$) and others ($n=4$). There were no patients with poorly-differentiated adenocarcinomas. As shown in Table 1, the relatively low expression level of MDR1 mRNA was suggested in moderately-differentiated adenocarcinomas (1.13±1.10) than well-differentiated (6.70±6.11, $p=0.111$).

To elucidate the association of MDR1 expression with differentiation, a colorectal adenocarcinoma cell line, HCT-15 was treated with two typical differentiating agents, NaB or ATRA, and as shown in Fig. 2, it was confirmed that MDR1 mRNA was up-regulated after their treatment.

Table 2 summarizes the effects of *MDR1* T-129C, G2677A,T and C3435T on the MDR1 mRNA expression in the colorectal adenocarcinomas and adjacent noncancerous colorectal tissues. The genetic polymorphism of C-145G was not detected in this study. The expression was lower in the patients with TC^{-129} than TT^{-129}, both in the colorectal adenocarcinomas ($p=0.040$) and adjacent noncancerous colorectal tissues ($p=0.023$), but their expression ratio was independent of T-129C ($p=0.149$). No such effects was found for G2677A,T and C3435T, but the comparison between variant carriers and non-carriers, *i.e.*, GG^{2677} *vs.* $GT^{2677}+AT^{2677}+TT^{2677}$ (no patients with GA^{2677} and AA^{2677} in this study) and CC^{3435} *vs.* $CT^{3435}+TT^{3435}$, resulted in lower values of the ratio in variant non-carriers; GG^{2677}: 0.18±0.09 *vs.* others^{2677}: 0.99±1.60 ($p=0.048$), CC^{3435}: 0.21±0.07 *vs.* $CT^{3435}+TT^{3435}$: 1.03±1.64 ($p=0.058$).

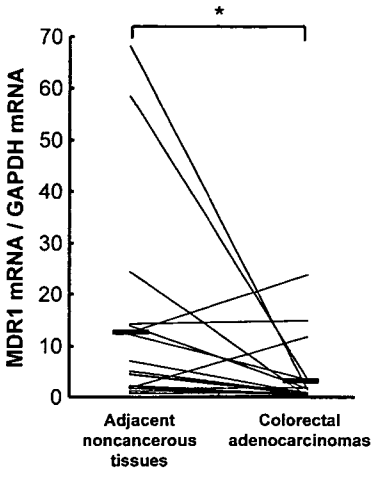

Fig. 1. Relative Concentrations of MDR1 mRNA in Colorectal Adenocarcinomas and Adjacent Noncancerous Colorectal Tissues Obtained from 21 Japanese Patients

Each bar represents the mean value. * $p=0.012$ by Wilcoxon signed-rank test.

DISCUSSION

With a number of clinical investigations, it has been demonstrated that a more preferable clinical response to chemotherapy is found for MDR1-negative tumors than MDR1-positive tumors.[9] Exposure to anticancer drugs sometimes results in the up-regulation of MDR1 in tumor tissues, and therefore in a poorer response when compared with pre-treatment.[9] Since MDR1, originally isolated from resistant tumor cells, appeared unique to sublines displaying an altered permeability to anticancer drugs, the MDR1 expression level-dependent response has been understood, in that MDR1 acts as an efflux pump exporting the anticancer drugs from the inside to the outside of the cells. Recent advances in cell biology have realized the concept of apoptosis to describe cellular events resulting in cell death. Smyth and Johnstone and their co-workers suggested that MDR1 protected cells

Table 1. Effects of Histological Type of Colorectal Adenocarcinomas on MDR1 mRNA Expression in Colorectal Adenocarcinomas and Adjacent Noncancerous Colorectal Tissues Obtained from 21 Japanese Patients

Histological type		Colorectal adenocarcinomas	Adjacent noncancerous colorectal tissues	Ratio[a]
Well-differentiated	5	6.70±6.11	17.73±23.27	1.53±2.59
Moderately-differentiated	12	1.13±1.10	11.97±20.07	0.62±1.10
Others	4	6.50±11.47	10.06±10.82	0.80±0.86
Total	21	3.48±5.97	12.98±18.85[b]	0.87±1.50

The values are the mean±S.D. *a*) Ratio = MDR1 mRNA in colorectal adenocarcinomas / MDR1 mRNA in adjacent noncancerous colorectal tissues. *b*) Higher than colorectal adenocarcinomas with statistical significance of $p=0.012$ by Wilcoxon signed-rank test.

Table 2. Effects of *MDR1* Genotypes of T-129C, G2677A,T and C3435T on MDR1 mRNA Expression in Colorectal Adenocarcinomas and Adjacent Noncancerous Colorectal Tissues Obtained from 21 Japanese Patients

			Colorectal adenocarcinomas		Adjacent noncancerous colorectal tissues		Ratio[a]	
T-129C	TT	18	3.95±6.34		14.68±19.90		0.96±1.61	
	TC	3	0.63±0.20	0.040[b]	2.75±1.76	0.023[b]	0.33±0.30	0.149
G2677A,T	GG	3	2.79±1.98		17.38±15.97		0.18±0.09	
	GT	13	3.36±6.81		10.10±18.53		1.21±1.84	
	AT+TT	5	4.19±5.99	0.949	17.82±23.41	0.694	0.43±0.42	0.448
C3435T	CC	4	1.23±1.41		5.32±4.69		0.21±0.07	
	CT	13	3.12±6.30		17.43±22.84		0.77±1.11	
	TT	4	6.89±7.35	0.403	6.19±5.86	0.407	1.88±2.85	0.283
Total		21	3.48±5.97		12.98±18.85		0.87±1.50	

The values are the mean±S.D. *a*) Ratio=MDR1 mRNA in colorectal adenocarcinomas / MDR1 mRNA in adjacent noncancerous colorectal tissues. *b*) Statistically significant difference was found between TT^{-129} and TC^{-129} ($p<0.05$), but no such difference was found on the genotypes of G2677A,T and C3435T.

against the caspase-dependent apoptosis induced by cytotoxic drugs, Fas ligation, tumor necrosis factor and ultraviolet irradiation.[25,26] We also found that MDR1 expression up-regulated by apoptotic stimuli suppressed caspase-dependent apoptotic signaling, presumably *via* a mitochondrial pathway.[23] Although the role of MDR1 in apoptosis has sometimes been discussed from the viewpoints of the sphingomyelin-ceramide pathway, acidification of the intracellular space, cholesterol esterification and cytokine release from lymphocytes,[11,12] these results are also consistent with the clinical observations that the higher expression of MDR1 results in a poorer response to chemotherapy.

Compared with apoptosis, relatively less information is available concerning the role of MDR1 in differentiation. Recently, we[23] and Goto *et al.*[27] have found that MDR1 mRNA is down-regulated in a human colon carcinoma cell line, Caco-2, prior to the up-regulation of villin mRNA, a marker of differentiation. Here, as shown in Fig. 1, it has been demonstrated that the expression level of MDR1 mRNA was lower in colorectal adenocarcinomas than adjacent noncancerous colorectal tissues ($p=0.012$). Lower levels of MDR1 in cancerous tissues than the adjacent normal tissues were also reported in French patients with renal cell carcinoma[28] and Japanese patients with colorectal carcinoma,[29] but the opposite result was obtained in French patients with advanced breast carcinoma.[30] A lower level of MDR1 mRNA in adenocarcinomas than adjacent noncancerous tissues suggests its down-regulation as a consequence of the malignant transformation of colorectal tissues, possibly with the suppression of differentiation. Potocnik *et al.* indicated a lower MDR1 expression in poorly-differentiated colorectal cancers obtained from Slovenia patients than well-differentiated cancers, with intermediate expression for moderately-differentiated cancers.[31] Poorly-differentiated types are found at 13.8—17.5% in Caucasians,[32,33] being more frequently found than the Japanese; that is, Takeuchi *et al.* reported poorly-, moderately- and well-differentiation types were found at 3.3%, 77.2% and 19.5% in adenocarcinomas, respectively,[16] suggesting a difference in the nature of the cancer between Caucasians and Japanese. In this study, a relatively low expression level of MDR1 mRNA was suggested in moderately-differentiated adenocarcinomas than in those that were well-differentiated, but there was no statistically significant difference ($p=0.111$). To elucidate the association of MDR1 expression with differentiation, a colorectal adenocarcinoma cell line, HCT-15 was treated with two typical differentiating agents, NaB or ATRA,[34—37] and as shown in Fig. 2, it was confirmed that MDR1 mRNA was up-regulated after their treatment. Although these agents do not always induce MDR1, depending on the experimental conditions, including the type of cells,[34—37] MDR1 might be involved in the cellular events resulting in differentiation without malignant transformation. We could not obtain poorly-differentiated adenocarcinomas herein from 21 patients, as expected by the report on frequency by Takeuchi *et al.*,[16] and further investigations with relatively large number of patients should be undertaken, hopefully with poorly-differentiated adenocarcinomas, to confirm the role of MDR1 in differentiation.

In this study, the effects of genetic polymorphisms of *MDR1* on the expression levels of MDR1 mRNA was examined to present alternative marker of prognosis, especially

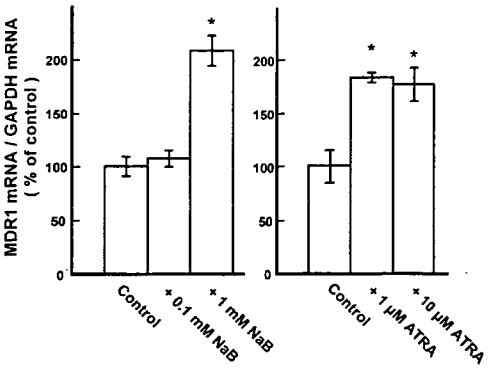

Fig. 2. Effects of Differentiating Agents on MDR1 mRNA Expression in Colorectal Adenocarcinoma Cell Line, HCT-15

(Left) Sodium butyrate (NaB), (Right) all-*trans* retinoic acid (ATRA). The cells were treated with NaB (0.1, 1 mM) or ATRA (1, 10 μM) for 24 h. For control cells, neither of NaB or ATRA was added. Each value represents the mean±S.D. of 5—7 independent experiments. * $p<0.05$, significantly different from the control.

when no extra biopsy samples will be obtained, based on the assumption that a lower expression level of MDR1 mRNA is associated with the suppression of differentiation and the acceleration of proliferation, resulting in the poor prognosis of patients with colorectal adenocarcinoma. A lower expression might induce the malignant transformation of colorectal tissues. Among more than 40 genetic polymorphisms, T-129C, G2677A,T and C3435T are often discussed in terms of their association with the expression,[1—5] and T-129C, but not G2677A,T or C3435T, was found to result in lower expression (Table 2). Such an effect of T-129C was also found in placentas obtained from Japanese people,[38] but we could not find it in duodenum biopsies obtained from healthy Japanese subjects.[21] The ratio of MDR1 mRNA expression in colorectal adenocarcinomas to adjacent noncancerous colorectal tissues was varied with an average of 0.87±1.50, and was independent of T-129C, G2677A,T and C3435T (Table 2). However, the comparison between variant carriers and non-carriers resulted in lower values in non-carriers of G2677A,T and C3435T, *i.e.*, GG^{2677} and CC^{3435}, than corresponding carriers, suggesting that MDR1 mRNA down-regulation after malignant transformation was more likely to be found in the non-carrier patients.

Collectively, the expression level of MDR1 mRNA was lower in colorectal adenocarcinomas than in the adjacent noncancerous colorectal tissues, suggesting its down-regulation as a consequence of the malignant transformation of colorectal tissues, possibly with the suppression of differentiation. A relatively lower expression was suggested in moderately-differentiated colorectal adenocarcinomas than well-differentiated adenocarcinomas. MDR1 mRNA up-regulation was found in HCT-15 cells after treatment with NaB and ATRA, suggesting its involvement in the cellular events resulting in differentiation without malignant transformation. *MDR1* T-129C, but not G2677A,T and C3435T, was associated with the lower expression of MDR1 mRNA both in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues, possibly being useful invasive marker predicting poorly-differentiated colorectal adenocarcinomas, and thereby, the poor prognosis of the patients, especially when no extra biopsy samples will be obtained. Further investigations with relatively large number of patients should be un-

dertaken to confirm these preliminary results.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Reserch from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by the YOKOYAMA Foundation for Clinical Pharmacology and by a research grant from Japan Research Foundation for Clinical Pharmacology.

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Drug Metab. Pharmacokinet. 21 (2): 126–132 (2006).

Regular Article

MDR1 Haplotype Frequencies in Japanese and Caucasian, and in Japanese Patients with Colorectal Cancer and Esophageal Cancer

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: The genotype frequencies of *MDR1* T-129C, C1236T, G2677A,T and C3435T SNPs were compared in 154 healthy Japanese and 100 healthy Caucasians to provide basic information on the inter-ethnic differences of pharmacotherapeutic outcome. The variants were found at allelic frequencies of 5.5%, 65.6%, 16.6%, 40.6% and 40.6%, for T-129C, C1236T, G2677A, G2677T and C3435T, respectively, in Japanese, and at 5.1%, 45.9%, 3.6%, 46.4% and 56.6%, respectively, in Caucasians, with a statistically significant difference for C1236T, G2677A,T and C3435T ($p<0.001$). G2677A was about 5-fold more frequent in Japanese than Caucasians. These genotype frequencies were also investigated in 95 Japanese patients with colorectal cancer (CRC) and esophageal squamous cell carcinoma (ESCC), but no significant difference was detected, when compared with healthy Japanese subjects. The haplotype frequency reached a total of about 85% in Japanese with the following 4 major haplotypes; T^{-129}-T^{1236}-T^{2677}-T^{3435} (36.1%), T^{-129}-T^{1236}-G^{2677}-C^{3435} (22.5%), T^{-129}-C^{1236}-G^{2677}-C^{3435} (14.2%) and T^{-129}-C^{1236}-A^{2677}-C^{3435} (13.3%). The second and fourth haplotypes were hardly inferred in Caucasian, whereas T^{-129}-C^{1236}-G^{2677}-T^{3435} (12.8%) was found to be Caucasian-specific. There was a tendency for higher frequencies of the T^{-129}/C^{-129}-C^{1236}-A^{2677}-C^{3435} haplotype in Japanese CRC patients and T^{-129}-T^{1236}-T^{2677}-T^{3435} haplotype in Japanese ESCC patients, compared with that in healthy Japanese subjects.

Key words: MDR1; genotype; haplotype; inter-ethnic difference; colorectal cancer; esophageal cancer

Introduction

Multidrug resistant transporter, MDR1/P-glycoprotein (ABCB1), the gene product of *MDR1*, was originally isolated from cancer cells that had developed resistance to anticancer drugs.[1-8] It has been elucidated that MDR1 is also expressed in normal tissues, including the liver, kidney, small and large intestines, brain, testis, muscle tissue, placenta, and adrenals, and confers an intrinsic resistance by exporting unnecessary and toxic exogenous substances or metabolites out of cells.[1-8] A number of structurally unrelated drugs have been found to be substrates for MDR1, and MDR1 and its related proteins are now recognized as important factors regulating the pharmacokinetics of drugs. Moreover, recent investigations have implicated MDR1 in the

Received; September 2, 2005, Accepted; November 23, 2005
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system regulating cell differentiation, proliferation and survival.

The first systemic screening for *MDR1* genetic polymorphisms was performed by Hoffmeyer *et al.* in 2000, and 15 single nucleotide polymorphisms (SNPs) were identified by analyzing 188 Caucasian individuals.[9] In the ensuring 5 years, several attempts have been made to identify additional SNPs and to examine their association with phenotypes. Recently, more than 40 SNPs were listed in an extensive examination by Kroetz *et al.*[10] Of these SNPs, a silent SNP in exon 26, C3435T, is the best characterized in terms of its association with the expression and/or function in the tissues, and also with pharmacokinetics and pharmacodynamics, however, there are still discrepancies in the results.[1-8]

Based on the assumption that MDR1 plays an important role in the detoxification systems of normal tissues, several studies have focused on the effects of C3435T on susceptibility to a certain class of disease. As for susceptibility to cancer, Siegsmund *et al.*[11] and Jamroziak *et al.*[12] suggested that the T^{3435}-allele is a risk factor for renal epithelial tumors and childhood acute lymphoblastic leukemia (ALL), respectively. C3435T has also reportedly been found more frequently in patients with colon cancer.[13-15] In contrast, Stanulla *et al.* suggested a significant reduction in the risk of relapse in the central nervous system in childhood ALL for patients with the T^{3435}-allele,[16] and Miller *et al.* reported no association with adult glioma.[17] Illmer *et al.* have reported *MDR1* genotype-related susceptibility to acute myeloid leukemia, where the heterozygote for C1236T, G2677T and C3435T was more frequently found among patients.[18] As stated above, there is no consensus on the association of C3435T with susceptibility to cancer, or with pharmacotherapy, indicating the need for a rational explanation and additional clinical investigations.

Herein, the genotype and haplotype frequencies of *MDR1* T-129C, C1236T, G2677A,T and C3435T SNPs were compared in 154 healthy Japanese and 100 healthy Caucasians to provide basic information on the inter-ethnic differences of pharmacotherapeutic outcome between both populations, and moreover were investigated in 95 Japanese patients with colorectal cancer (CRC) and esophageal squamous cell carcinoma (ESCC) to examine their potential as predictors of cancer susceptibility.

Materials and Methods

Subjects: A total of 95 unrelated Japanese patients with CRC (34 males and 14 females) and ESCC (44 males and 3 females) diagnosed at Kobe University Hospital participated in this study. Diagnoses of colorectal adenocarcinoma and esophageal carcinoma were based on clinical, endoscopic, radiologic, and histopathological findings as described previously.[19,20] The average age was 66.4±11.5 (±SD) years old (range, 28-82 years) and 64.8±8.0 years old (range, 48-83 years), respectively. One hundred and fifty-four unrelated healthy Japanese subjects (47 males and 107 females) aged 26.3±7.5 (range, 21-57) years were also enrolled and served as the healthy Japanese subjects. Written informed consent was obtained from all patients at the beginning of this study. The protocol for this study was approved by the Institutional Review Board of Kobe University Hospital, Japan. The data for MDR1 genotypes in 100 healthy Caucasians were quoted from a study which was performed as the Pharmacogenetics of Membrane Transporter Project at the University of California, San Francisco in the United States.[10]

Isolation of genomic DNA: Peripheral blood (2.0 mL) was drawn from the subject into a sampling tube containing EDTA-2Na (3.0 mg), and genomic DNA was extracted from 0.2 mL of whole blood using a QIAamp DNA Blood mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

MDR1 genotyping of T-129C, C1236T, G2677A,T and C3435T: The genetic polymorphisms T-129C, G2677A,T and C3435T were determined using the TaqMan® MGB probe and primer as reported previously.[21] To determine C1236T, the following probe and primers were designed using the software Assays-by-DesignSM (Applied Biosystems, Foster, USA): the C^{1236}-allele probe, 5′-CAG GTT CAG gCC CTT-3′; the T^{1236}-allele probe, 5′-TTC AGG TTC AGa CCC TT-3′; the forward primer, 5′-CAC CGT CTG CCC ACT CT-3′; and the reverse primer, 5′-GTG TCT GTG AAT TGC CTT GAA GTT T-3′. Lower-case font represents the SNP. As a reporter at the 5′ end of the TaqMan® MGB probe, VIC® was used for the C^{1236}-allele and 6-carboxyfluorescein (FAM) was used for the T^{1236}-allele. All TaqMan® MGB probes and primers used in this study were synthesized by Applied Biosystems Japan, Ltd. (Tokyo, Japan).

Haplotype analysis: The *MDR1* haplotypes were statistically inferred using an algorithm based on Bayesian inference, PHASE version 2.0.2 (http://www.stat.Washington.edu/stephens/) with a fair degree of precision.[10,22,23] PHASE calls were made separately for each ethnic and disease group. Haplotypes were inferred by running PHASE a total of 10 times, and relative standard deviation of their frequencies was 5% or less of the mean value. The estimated haplotype frequencies were expressed as the average population haplotype frequencies for the whole sample (referred to as the "Population haplotype") as well as the practical haplotype frequencies based on the most likely inferred pairs of haplotypes identified at least 8 of 10 times for each individual (referred to as the "Practical haplo-

Table 1. Allelic and genotype frequencies of *MDR1* T-129C, C1236T, G2677A,T and C3435T in Japanese and Caucasians, and in Japanese patients with colorectal cancer and esophageal cancer.

SNP	Subject[a]	N	Allele				Genotype						
T-129C			T	C		*P*-value[b]	TT	TC	CC				*P*-value[b]
	Japanese Healthy	154	291 94.5%	17 5.5%			139 90.3%	13 8.4%	2 1.3%				
	CRC	48	89 92.7%	7 7.3%		0.620	41 85.4%	7 14.6%	0 0.0%				—
	ESCC	47	88 93.6%	6 6.4%		0.800	41 87.2%	6 12.8%	0 0.0%				—
	Caucasian Healthy[c]	99	188 94.9%	10 5.1%		1.000	89 89.9%	10 10.1%	0 0.0%				—
C1236T			C	T			CC	CT	TT				
	Japanese Healthy	154	106 34.4%	202 65.6%			17 11.0%	72 46.8%	65 42.2%				
	CRC	48	38 39.6%	58 60.4%		0.393	7 14.6%	24 50.0%	17 35.4%				0.618
	ESCC	47	39 41.5%	55 58.5%		0.222	6 12.8%	27 57.4%	14 29.8%				0.299
	Caucasian Healthy[c]	97	105 54.1%	89 45.9%		<0.001	29 29.9%	47 48.5%	21 21.6%				<0.001
G2677A,T			G	A	T		GG	GA	GT	AA	AT	TT	
	Japanese Healthy	154	132 42.9%	51 16.6%	125 40.6%		30 19.5%	23 14.9%	49 31.8%	4 2.6%	20 13.0%	28 18.2%	
	CRC	48	36 37.5%	20 20.8%	40 41.7%	0.528	8 16.7%	5 10.4%	15 31.3%	2 4.2%	11 22.9%	7 14.6%	—
	ESCC	47	39 41.5%	14 14.9%	41 43.6%	0.867	8 17.0%	5 10.6%	18 38.3%	0 0.0%	9 19.1%	7 14.9%	—
	Caucasian Healthy[c]	98	98 50.0%	7 3.6%	91 46.4%	<0.001	27 27.6%	3 3.1%	41 41.8%	1 1.0%	2 2.0%	24 24.5%	—
C3435T			C	T			CC	CT	TT				
	Japanese Healthy	154	183 59.4%	125 40.6%			55 35.7%	73 47.4%	26 16.9%				
	CRC	48	56 58.3%	40 41.7%		0.905	14 29.2%	28 58.3%	6 12.5%				0.411
	ESCC	47	50 53.2%	44 46.8%		0.286	11 23.4%	28 59.6%	8 17.0%				0.255
	Caucasian Healthy[c]	99	86 43.4%	112 56.6%		<0.001	21 21.2%	44 44.4%	34 34.3%				0.003

[a]CRC: Colorectal cancer, ESCC: Esophageal squamous cell carcinoma
[b]Allelic and Genotype frequency comparisons with Japanese healthy subjects (Fisher's exact test).
[c]Data from the study reported by Kroetz *et al.*[10]

type'').

Statistical analysis: Differences in genotype and allelic frequencies between healthy Japanese and Caucasian subjects and between the healthy subjects and patients in Japan were examined using Fisher's exact statistical tests. P values of less than 0.05 were considered significant.

Results

Table 1 lists the *MDR1* allelic and genotype frequencies determined in healthy Japanese and Caucasian subjects, and in Japanese patients with CRC and ESCC. In healthy Japanese subjects, the variants were found at allelic frequencies of 5.5%, 65.6%, 16.6%, 40.6% and 40.6%, for T-129C, C1236T, G2677A, G2677T and

Table 2. Estimated frequencies of *MDR1* haplotypes in Japanese and Caucasians, and in Japanese patients with colorectal cancer and esophageal cancer.

Position				Nomenclature[a]	Estimated Frequency (%)[b]			
					Japanese			Caucasian[c,d] Healthy
−129	1236	2677	3435		Healthy N=154	CRC N=48	ESCC N=47	N=95
T	T	T	T	**13/*13A/*14/*14A/*16*	36.1 (36.4)	37.1 (37.5)	42.3 (42.6)	40.0 (41.1)
T	T	G	C	**11/*11A/*11B/*19*	22.5 (22.4)	17.8 (16.7)	15.3 (14.9)	1.0 (1.1)
T	C	G	C	**1/*8/*8A/*9/*9A/*9B/*9C/ *10/*20/*21/*21A/*21B/*21E/ *22/*22A/*23/*23A/*25/*26/ *26A/*26B/*26D/*27/*28/ *29/*30/*31/*32*	14.2 (14.6)	15.7 (16.7)	19.5 (20.2)	32.5 (32.6)
T	C	A	C	**24/*24A*	13.3 (13.0)	14.6 (14.6)	10.8 (10.6)	1.6 (1.6)
T	T	T	C	**15/*15A*	4.0 (3.9)	3.2 (3.1)	0.6 (1.1)	3.1 (2.6)
C	C	A	C	**4A*	3.1 (3.6)	6.1 (6.3)	3.9 (4.3)	<0.1 (−)
T	T	G	T	**12/*12A/*18/*19A*	2.5 (2.6)	1.9 (3.1)	<0.1 (−)	0.5 (0.5)
C	C	G	C	**3/*4/*4B/*5/*5A/*6/ *6A/*7*	1.9 (1.6)	0.5 (1.0)	2.4 (2.1)	3.7 (4.2)
T	C	G	T	**2/*2A/*17/*21C*	1.5 (1.3)	1.0 (−)	4.2 (4.3)	12.8 (12.1)
C	T	G	C	*not assigned*	0.4 (0.3)	0.2 (−)	<0.1 (−)	<0.1 (−)
T	C	T	T	**17A*	0.3 (0.3)	1.2 (1.0)	<0.1 (−)	1.0 (1.1)
C	C	T	T	*not assigned*	0.1 (−)	<0.1 (−)	<0.1 (−)	<0.1 (−)
T	C	T	C	**21D/*26C*	<0.1 (−)	<0.1 (−)	0.5 (−)	1.7 (1.6)
T	C	A	T	**2B*	<0.1 (−)	<0.1 (−)	<0.1 (−)	1.0 (1.1)
C	C	G	T	*not assigned*	<0.1 (−)	0.3 (−)	<0.1 (−)	0.5 (0.5)
C	T	G	T	**12B*	<0.1 (−)	0.1 (−)	<0.1 (−)	<0.1 (−)
C	T	A	T	*not assigned*	<0.1 (−)	<0.1 (−)	0.1 (−)	<0.1 (−)
C	T	T	T	*not assigned*	<0.1 (−)	<0.1 (−)	<0.1 (−)	0.5 (−)

[a]Allele assigned by Kroetz *et al.*[10] based on 64 distinct haplotypes obtained for 28 variant sites in a total of 494 subjects. Sixty-three were inferred for either of the groups, but *MDR1*15B* (C^{-129}-T^{1236}-T^{2677}-C^{3435}) was not detected.
[b]"Population haplotype" frequencies are presented with "Practical haplotype" frequencies in parentheses. —: Not inferred.
[c]Haplotype frequencies were calculated in 95 healthy Caucasian subjects.
[d]Data from the study reported by Kroetz *et al.*[10]

C3435T respectively. For healthy Caucasians, they were found at 5.1%, 45.9%, 3.6%, 46.4% and 56.6%, respectively, with a statistically significant difference for C1236T, G2677A,T and C3435T ($p<0.001$). It is noted that G2677A was about 5-fold more frequently found in Japanese than Caucasian. Statistically significant differences were also detected for their genotypes (**Table 1**). The variants were found at frequencies of

7.3%, 60.4%, 20.8%, 41.7% and 41.7% for 48 Japanese CRC patients and 6.4%, 58.5%, 14.9%, 43.6% and 46.8% for 47 Japanese ESCC patients, respectively. No significant difference was detected for allelic and genotype frequencies between healthy Japanese subjects and either group of cancer patients.

Table 2 shows the estimated frequencies of *MDR1* haplotypes in healthy Japanese and Caucasian subjects, and in Japanese patients with CRC and ESCC. No difference in frequency was found between the "Population haplotype" and "Practical haplotype". In healthy Japanese subjects, 12 haplotypes were statistically inferred with a frequency of more than 0.1% at the population base. With the major 4 haplotypes, the frequency reached a total of about 85%; T^{-129}-T^{1236}-T^{2677}-T^{3435} (36.1%), T^{-129}-T^{1236}-G^{2677}-C^{3435} (22.5%), T^{-129}-C^{1236}-G^{2677}-C^{3435} (14.2%) and T^{-129}-C^{1236}-A^{2677}-C^{3435} (13.3%). T^{-129}-T^{1236}-G^{2677}-C^{3435} (1.0%) and T^{-129}-C^{1236}-A^{2677}-C^{3435} (1.6%) were rare, and 3 of 12 haplotypes found in Japanese were not statistically inferred with a frequency of more than 0.1% in Caucasians, whereas T^{-129}-C^{1236}-G^{2677}-T^{3435} (12.8%) was Caucasian-specific. There was no significant difference in the estimated frequencies of *MDR1* haplotypes between healthy Japanese subjects and Japanese CRC or ESCC patients, although there was a tendency for higher frequencies of the T^{-129}/C^{-129}-C^{1236}-A^{2677}-C^{3435} haplotype in CRC patients and T^{-129}-T^{1236}-T^{2677}-T^{3435} haplotype in ESCC patients, compared with that in healthy Japanese subjects (**Table 1**).

Discussion

In this examination, the frequency of variants T-129C, C1236T, G2677A, G2677T and C3435T in healthy Japanese subjects was 5.5%, 65.6%, 16.6%, 40.6% and 40.6%, respectively (**Table 1**). These results are consistent with other studies on Japanese and our previous report with a smaller population, i.e., 6.0–9.3% for T-129C,[21,24-26] 55.5–61.5% for C1236T,[24,25] 16.9–20.5% for G2677A,[21,24-26] 35.5–40.8% for G2677T,[21,24-26] and 38.5–44.1% for C3435T.[21,24-27] In Caucasians, the allelic frequencies were found to be 5.1%, 45.9%, 3.6%, 46.4% and 56.6%, respectively, again consistent with previous reports, i.e., 5.1–5.9% for T-129C,[9,10] 37.8–45.9% for C1236T,[9,10,28,29] 1.9–3.6% for G2677A,[10,28,29] 41.6–46.4% for G2677T,[10,28,29] and 48.0–56.1% for C3435T.[9,10,28,29] Inter-ethnic differences in frequencies have been studied extensively for C3435T among SNPs ever since the report by Hoffmeyer *et al.* on the effects of *MDR1* genotype showed the association of C3435T with the expression of MDR1 in the duodenum, and thereby with the pharmacokinetics of digoxin, a typical MDR1 substrate, after oral administration.[9] In the past 5 years, a number of clinical studies have been performed to replicate these findings; however, there are still discrepancies, suggesting the effects of other SNPs.[1-8] The allelic and genotype frequencies for T-129C, C1236T and G2677A,T presented herein might be useful for explaining the differences with the findings of Hoffmeyer *et al.*[9]

MDR1 is understood to play an important role in the detoxification systems of normal tissues, and C3435T has been evaluated in terms of disease susceptibility. Several reports have suggested that this SNP is a risk factor for cancer, including colorectal cancer,[11-15] although this is not always the case.[16,17] Here, it was found that C3435T was not associated with CRC or ESCC in Japanese. Additionally, the effects of 3 other SNPs were evaluated. Although there was a tendency for a higher frequency of the A^{2677}-allele in the CRC patients, the T^{3435}-allele in the ESCC patients, and TC^{-129}, CT^{1236}, AT^{2677} and CT^{3435} genotypes in the CRC and ESCC patients, compared to healthy Japanese subjects, the study was not sufficiently powered to reach statistical significance (**Table 1**). In Japan, the incidence of CRC and ESCC is increasing in Japan,[30] and therefore further studies are needed to examine whether environmental factors, unexamined *MDR1* genotypes, and/or genes other than *MDR1* gene, were more predominant for the development of CRC and ESCC.

To explain the diversity of the results of C3435T, several reports suggested the importance of linkage disequilibrium of C3435T with C1236T and G2677T, the latter resulting in Ala893Ser, that is, C3435T may not itself be causal but rather may be linked with the causal polymorphisms.[1-8] Johne *et al.* defined 4 haplotypes; 11, 12, 21 and 22, and 9 genotypes; 00, 01, 02, 10, 11, 12, 02, 21 and 22, based on G2677T and C3435T,[31] where the haplotype coding is as follows; 1: identical to the reference sequence (G^{2677}/C^{3435}); 2: different from it (T^{2677}/T^{3435}), and therefore the genotype coding is as follows; 0: homozygous for nucleotides identical to the reference sequence for the position on both chromosomes; 1: heterozygous; 2: homozygous for nucleotides different from the reference sequence for the position on both chromosomes. For genotype 11, 11/22, not 12/21, is selected based on the frequency in Caucasians. This assignment is often used to check the importance of haplotype analyses,[1-8] but it is noted that a variant A^{2677}-allele was preferentially found in Japanese as shown in **Table 1**. This allele has been shown to be important for the pharmacokinetics of the H_1-antihistamine, fexofenadine,[32] and thus an analysis based on Johne's assignment is insufficient for Japanese. Haplotype may often provide more useful information than genotype about inter-individual and inter-ethnic differences of pharmacokinetics and pharmacodynamics.[33] Kim *et al.*,[29] Sai *et al.*,[24] and Tang *et al.*[34] have defined 11 haplotypes, *MDR1*1* to **11*, and their subtypes based on C1236T, G2677A,T and C3435T, but Saito

et al. have suggested the importance of T-129C.[21] Kroetz *et al.* defined 32 haplotypes, *MDR1*1* to *MDR1*32*, and their subtypes (a total of 64 haplotypes).[10] These assignments should be justified by genotype-phenotype correlation studies. As shown in **Table 2**, there is a considerable difference in the frequencies of haplotype including A^{2677} polymorphism between Japanese and Caucasian. This might contribute in part to the inter-ethnic discrepancies of the results on C3435T.

Estimates of *MDR1* haplotype frequencies showed that 3 of 12 haplotypes found in Japanese were hardly inferred in Caucasians, whereas T^{-129}-C^{1236}-G^{2677}-T^{3435} was Caucasian-specific. A tendency for higher frequencies of the T^{-129}/C^{-129}-C^{1236}-A^{2677}-C^{3435} haplotype in the CRC patients and the T^{-129}-T^{1236}-T^{2677}-T^{3435} haplotype in the ESCC patients was also observed in comparison with that in the healthy Japanese subjects. These results suggested that *MDR1* haplotypes based on 4 sites, −129, 1236, 2677 and 3435, may be able to be used to characterize ESCC and CRC patients in the Japanese population, and future large scale studies are warranted to appropriately investigate this possibility.

Acknowledgements: This work was supported in part by a Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The Caucasian genotyping data was collected as part of the Pharmacogenetics of Membrane Transporter project funded by grant GM61390 from the National Institutes of Health.

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Journal of Gastroenterology and Hepatology (2006) 21, 420–424 DOI: 10.1111/j.1440-1746.2005.04112.x

GASTROENTEROLOGY

HLA-DQB1 locus and gastric cancer in *Helicobacter pylori* infection

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Abstract

Background and Aims: It has been suggested that the incidence of digestive diseases associated with *Helicobacter pylori* is influenced by the strain diversity of *H. pylori*, factors involving the host or environment, and the duration of infection. The authors have previously reported that human leukocyte antigen (HLA)-DQB1*0401 plays an important role in the development of atrophic gastritis in *H. pylori* infected patients. The aim of the present study was to investigate the relationship between HLA-DQB1 genotype and cancer development.

Methods: HLA-DQB1 genotyping was performed by the PCR-RFLP method on 122 *H. pylori*-infected non-ulcer dyspepsia (NUD) patients, 53 gastric cancer patients and 28 uninfected controls. To reliably estimate the grade of atrophic gastritis, histological evaluation was performed.

Results: The allele frequency of DQB1*0401 was significantly higher in intestinal type cancer patients compared with age- and sex-matched *H. pylori*-infected NUD patients. There was no significant difference in the mean atrophic scores of the biopsy samples from the lesser curvature of the mid-corpus between these groups.

Conclusions: HLA-DQB1*0401 is a useful marker for determining susceptibility to intestinal type gastric cancer.

Key words: gastric cancer, *Helicobacter pylori*, HLA-DQB1.

INTRODUCTION

Helicobacter pylori is known to be involved in peptic ulcer[1] and the development of atrophic gastritis,[2] and was suggested to be a risk factor for gastric cancer.[3–6] Although more than 50% of the Japanese population is infected with *H. pylori*, only a small percentage of the infected population suffers from peptic ulcers or gastric cancer. This discrepancy may be due to the strain diversity of *H. pylori*, factors involving the host or environment, and the duration of infection. Little is known about the relationship between these factors and *H. pylori*-related diseases. Our previous study revealed that human leukocyte antigen (HLA)-DQB1*0401 plays an important role in the development of atrophic gastritis in *H. pylori* infected patients.[7] Other previous investigations have linked specific HLA class II alleles to cancer development.[8–11]

Human leukocyte antigen class II molecules are α–β heterodimeric membrane glycoproteins that are expressed on the surface of antigen-presenting cells such as macrophages, dendritic cells and B lymphocytes.[12] Helper T cells can only recognize peptides, derived from extracellular antigens, that are associated with HLA class II molecules. The interaction of T cell receptors, peptides and HLA class II molecules determines T cell activation and an immune response to antigens.[13] HLA polymorphism is responsible for variations in the immune response of different individuals to different antigens, and contributes to the susceptibility or resistance to infectious and autoimmune diseases.[14–18] HLA class II genes are

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Accepted for publication 17 January 2005.

contained in the HLA-D region, which spans about 1100 kilobases of the short arm of chromosome 6. The HLA-D region contains three principal subregions, DP, DQ, and DR. With respect to gastric cancer, it has been reported that, although HLA-DQB1*0602 and DRB1*1601 are positively associated with gastric adenocarcinoma,[8,9] DQA1*0102 is negatively associated,[10] and controversy exists regarding HLA-DQB1*0301.[8,11] In the present study, we investigated if the HLA-DQB1 locus has an influence on gastric cancer susceptibility.

METHODS

Fifty-three patients with gastric adenocarcinoma, 122 *H. pylori*-infected non-ulcer dyspepsia (NUD) patients and 28 uninfected controls were studied (Table 1). All subjects were Japanese. NUD patients were endoscopically diagnosed to have atrophic gastritis, and those with limited lesions such as peptic ulcer or gastric cancer or gastric lymphoma of mucosa-associated lymphoid tissue (MALT) were excluded. Patients taking ulcerogenic drugs such as steroids or non-steroidal anti-inflammatory drugs were also excluded. Written informed consent was obtained from all subjects in this study.

Helicobacter pylori infection was diagnosed by culturing two biopsy samples (the greater curvature of the antrum and mid-corpus), microscopic examination (Warthin–Starry stain) of four biopsy samples (the greater curvature of the antrum and mid-corpus and the lesser curvature of the antrum and mid-corpus) and *H. pylori* antibody immunoglobulin G. Subjects were considered infected with *H. pylori* if two or more of these tests were positive (the antibody test is necessarily positive) and uninfected if all three tests were negative.

The grade of atrophic gastritis was estimated by histological evaluation (hematoxylin and eosin stain) at the lesser curvature of the mid-corpus according to the Updated Sydney System (0, normal; 1, mild; 2, moderate; 3, marked;).[19]

Genomic DNA was obtained from peripheral blood leukocytes and HLA-DQB1 genotype was defined by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method.[20] PCR primers for amplification and restriction endonuclease for genotyping of DQB1 alleles were as described previously.[7]

The frequencies of HLA-DQB1 alleles were analyzed by the chi-squared and Fisher's exact tests. The significance of the difference in histological atrophic score was determined by Student's *t*-test for unpaired samples. *P*-values of <0.05 were considered significant.

RESULTS

Table 1 shows the clinical background of gastric cancer patients, *H. pylori*-infected NUD patients and uninfected controls. Cancer patients were all infected with *H. pylori*. The allele frequency of DQB1*0401 was significantly higher in cancer patients compared with *H. pylori*-infected NUD patients, resulting in an odds ratio of 2.83 (95% confidence interval = 1.44–5.55, $P < 0.005$)(Fig. 1a, Table 2). The allele frequency of DQB1*0401 was significantly different between these groups also after Bonferroni's correction ($P < 0.05$). However, there was a significant difference in the mean age between these groups.

As atrophic gastritis has been suggested to be the precursor lesion of intestinal type gastric adenocarcinoma, we classified the NUD group according to age ≥55 years to exclude the influence of age (Table 1) and compared this sub-group to the intestinal type cancer patients. The allele frequency of DQB1*0401 was significantly higher in intestinal type cancer patients (52.8%) compared with NUD patients aged 55 years and above (30.6%), resulting in an odds ratio of 2.53 (95% confidence interval = 1.04–6.19, $P < 0.05$). (Fig. 1b, Table 2). However, the allele frequency of DQB1*0401 was not significantly different between these groups after Bonferroni's correction ($P < 0.1$). There was no significant difference in the mean age, sex ratio or histological evaluation of atrophy between these groups (Table 1).

The allele frequency of DQB1*0302 was significantly higher in diffuse type cancer patients compared with NUD patients, resulting in an odds ratio of 5.83 (95% confidence interval = 2.01–16.9, $P < 0.005$) (Table 2). However, the number of diffuse type cancer cases was low, and an additional study is required to investigate this further.

There were no other significant differences among frequency of alleles.

Table 1 Clinical background of *Helicobacter pylori*-infected patients with either gastric cancer or non-ulcer dyspepsia compared to uninfected controls

	Gastric cancer		Non-ulcer dyspepsia		
Characteristic	Intestinal	Diffuse	All	Age ≥55 years	Control
No. patients (Male/Female)	36 (20/16)	17 (10/7)	122 (78/44)	49 (24/25)	28 (17/11)
Age (years) [mean ± SD]	66.9 ± 10.5	55.8 ± 11.0	50.9 ± 14.0	64.4 ± 6.5	43.0 ± 16.8
Age (years) [range]	30–84	34–69	16–80	55–80	21–78
Atrophy [mean ± SD]	1.6 ± 0.9	1.1 ± 0.9	1.3 ± 1.2	1.7 ± 1.2	0.2 ± 0.7

HP, *Helicobacter pylori*.

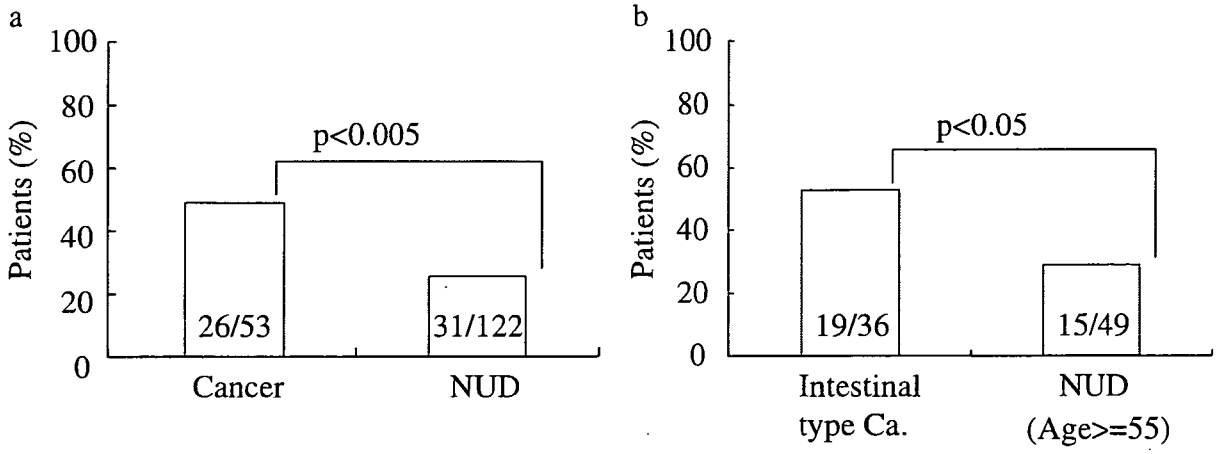

Figure 1 (a) Frequency of HLA-DQB1*0401 in *Helicobacter pylori*-infected patients with either gastric cancer or non-ulcer dyspepsia (NUD). (b) Frequency of HLA-DQB1*0401 in *Helicobacter pylori*-infected patients with either intestinal type cancer or non-ulcer dyspepsia (NUD) aged 55 years and above.

Table 2 Frequency of HLA-DQB1 alleles in *Helicobacter pylori*-infected patients with either gastric cancer or non-ulcer dyspepsia compared to uninfected controls

HLA-DQB1 allele	Gastric cancer (%)		Non-ulcer dyspepsia (%)		
	Intestinal	Diffuse	All	Age ≥55 years	Control (%)
0201	0	5.9	0	0	0
0301	2.8	5.9	21.3	10.2	21.4
0302	33.3	58.8[c]	19.7[c]	28.6	17.9
0303	11.1	0	23.0	24.5	25.0
0401	52.8[a,b]	41.2	25.4[a]	30.6[b]	7.1
0402	8.3	0	6.6	6.1	7.1
0501	2.8	0	14.8	14.3	17.9
0502	0	5.9	4.9	2.0	3.6
0503	13.9	5.9	8.2	12.2	10.7
0504	0	0	0	0	0
0601	36.1	52.9	31.1	30.6	32.1
0602–0603	19.4	23.5	10.9	20.4	14.3
0604–0605	19.4	0	13.9	8.2	17.9

[a,c]$P < 0.005$; [b]$P < 0.05$.

DISCUSSION

Human leukocyte antigen class II plays a pivotal role in the immune response against foreign antigens. Therefore, it is important to analyze HLA for susceptibility or resistance to disease affected by *H. pylori* infection. Previous studies showed that HLA-DQA1 genotypes contribute to peptic ulcer in *H. pylori* infection,[21] and that HLA-DQ types affect progression to atrophy in *H. pylori* infection.[22] Azuma *et al.*[21] reported that DQA1*0102 might contribute to resistance against *H. pylori* associated gastric atrophy and its association with intestinal type gastric adenocarcinoma. Magnusson *et al.*[9] reported that DRB1*1601 was associated with gastric adenocarcinoma. We reported the DQB1*0401 plays an important role in the development of atrophic gastritis in *H. pylori* infected patients.[7] Although Lee *et al.*[11] reported that DQB1*0301 was positively associated with gastric adenocarcinoma, Wu *et al.*[8] reported it was negatively associated. In the present study, DQB1*0301 was less common in cancer patients than in NUD patients and uninfected controls. We speculate this discrepancy may be caused by difference in ethnic background, as the frequency of HLA class II alleles differs markedly between races.[23]

Helicobacter pylori infection is a significant risk factor for the development of atrophic gastritis and intestinal metaplasia,[24] and intestinal metaplasia is associated with increased risk for gastric carcinomas.[25] Atrophic gastritis was suggested to be the precursor lesion of the intestinal type gastric adenocarcinoma, so it is postulated that HLA-DQB1*0401 would also influence cancer susceptibility. It is suggested that immunogenic factors play an important role in the development of

atrophic gastritis and gastric adenocarcinoma susceptibility in *H. pylori*-infected patients. The present study reveals that HLA-DQB1*0401 is a marker not only for the development of atrophic gastritis but also for the intestinal type gastric adenocarcinoma itself. Although it is useful that the association remains significant after Bonferroni's correction (Fig. 1b, Table 2), Bonferroni's correction for multiple inferences is not strictly required for the chi-squared analysis between HLA-DQB1*0401 and gastric cancer because this association was particularly sought in the current study. Concerning DQB1*0401, a positive association in duodenal ulcers was reported in Japan,[26] but gastric cancer patients were not investigated in that study. The finding that the frequency of HLA class II alleles differs markedly between races should be considered.[23] Therefore, DQB1*0401-positive Japanese with *H. pylori* infection may have a particular immune response to antigens and be susceptible to these diseases.

The 3-D structure of the HLA class II DR1 molecule has been determined by X-ray crystallography and is similar to that of the HLA class I molecule.[27] Both HLA class I and class II molecules are composed of two membrane-proximal immunoglobulin-like domains and a membrane-distal peptide-binding site formed by an eight-stranded β-sheet and two α-helical regions and have polymorphic pockets that accommodate side chains of peptides in the peptide-binding site.[27–29] It was suggested that a particular amino acid at position 57 of the HLA-DQB chain contributes to susceptibility and resistance to insulin-dependent diabetes mellitus.[16] The allele frequency of DQB1*0401 is significantly higher in intestinal type cancer patients than that in dyspepsia patients, but in the allele frequency of DQB1*0402 there is no difference. DQB1*0401 differs from DQB1*0402 by only a single amino acid at position 86 of the HLA-DQB chain (0401 glycine). This position is important to determine the 3-D structure of the DQ molecule, that is, to form a pocket that accommodates a side chain of peptides, similar to position 57 of the HLA-DQB chain. It is suggested that glycine at this position plays an important role in susceptibility to the development of atrophic gastritis and gastric cancer.

Therefore, it is suggested that DQB1*0401 is a useful marker for determining susceptibility to atrophic gastritis and intestinal type gastric cancer. However, about a half of gastric cancer patients do not have DQB1*0401 in this study. It has been suggested that gastric cancer is a multifactorial inherited disease, and future investigations should seek to define potential associations between HLA class II alleles and gastric cancer.

Recently it was discussed whether asymptomatic individuals with *H. pylori* infection should have their infection eradicated. Epidemiological evidence suggests that *H. pylori* infection increases the risk for gastric carcinoma.[30–33] In Mongolian gerbils, long-term infection with *H. pylori* induced adenocarcinoma.[34,35] In established experimental models of stomach carcinogenesis in Mongolian gerbils it has been demonstrated that *H. pylori* infection exerts an enhancing effect on tumor development in animals treated with a chemical carcinogen.[36] Eradication therapy diminishes the enhancing effects of *H. pylori* infection on glandular stomach carcinogenesis in Mongolian gerbils.[37] Further, the reduced likelihood of metachronous cancer development and growth inhibition in individuals by *H. pylori* eradication was reported.[38]

Eradication therapy is advisable in *H. pylori*-positive patients with a family history of gastric cancer.[39] Due to the high costs of eradication therapy, it is impossible to eradicate infection in all *H. pylori*-positive patients; therefore, the identification of the high-risk group for eradication is very important. We suggest that DQB1*0401-positive individuals with *H. pylori* infection should have their infection eradicated and, because they are significantly susceptible to gastric cancer, it is necessary that they are observed carefully using follow-up endoscopy.

ACKNOWLEDGMENTS

The authors express great thanks to Miss Satomi Shigeta and Mr Ken Yoshimura for excellent assistance in data collection.

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