quence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for the MDR1, LRP, and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes were as follows. The sequence of the forward primer for MDR1 mRNA was 5′-GTCTACAGTTCGTAATGCTGACGT-3′ and that of the reverse primer was 5′-TGTGATCCACGGACACTCCTAC-3′; the TaqMan probe used was 5′-CGCTGGTTTCGATGATGGAGTCATTG-3′. For LRP mRNA, the forward primer was 5′-CGCTGTGATTGGAAGCACCTA-3′ and the reverse primer was 5′-CGGGAGGCAGCTCTTTCTC-3′; the TaqMan probe was 5′-ATGCTGACCCAGGACGAAGTCCT-3′. The sequence for the forward primer for survivin mRNA was 5′-TGCCTGGCAGCCCTTTC-3′ and for the reverse primer was 5′-CCTCCAAGAAGGGCCAGTTC-3′; for the TaqMan probe, it was 5′-CAAGGACCACCGCATCTCTACATTC-3′. For cIAP1 mRNA, the sequence for the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and for the reverse primer, it was 5′-CAAGCCACCATCACAACAAAA-3′; for the TaqMan probe, it was 5′-TTTATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, the sequences of the forward and reverse primer were 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and 5′-TCTCCTGGGCTGTCTGATGTG-3′, respectively; and the sequence for the TaqMan probe was 5′-CCCTCATCTACTTGAACAGCTGCTAT-3′. The forward and reverse sequences for NAIP mRNA were 5′-GCTTCACAGCGCATCGAA-3′ and 5′-GCTGGGCGGATGCTTTC-3′, respectively; while the sequence for the TaqMan probe was 5′-CCATTTAAACCACAGCAGAGGCTTTAT-3′. The sequence of the forward primer for XIAP mRNA was 5′-AGTGGTAGTCCTGTTTCAGCATCA-3′ and for the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the sequence for the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTTTATACTG-3′. Finally, the forward primer sequence for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGAGT-3′ and for the reverse primer was 5′-GAAGATGGTGATGGGATTTC-3′; the TaqMan probe sequence was 5′-CAAGCTTCCCGTTCTCAGCC-3′. The conditions for one-step RT-PCR were as follows: 2 min at 50°C (Stage 1, reverse transcription), 10 min at 95°C (Stage 2, RT inactivation and AmpliTaq Gold activation), and then 45 cycles of amplification for 15 sec at 95°C and 1 min at 60°C (Stage 3, PCR). The expression of MDR1, LRP, or IAP family proteins was quantitated according to a method described elsewhere [23]. Briefly, the intensity of the reaction was evaluated from the quantity of total RNA in Raji cells (ng) corresponding to the initial number of PCR cycles to reveal the linear increase in reaction intensity (threshold cycle) for each sample on a logarithmic standard curve. Data on the quantity of RNA (ng) for the MDR1, LRP, and IAPs were normalized using the data for GAPDH in each sample.

Identification of Apoptotic Cells

To identify apoptotic cells, the terminal deoxytransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was employed as previously described [24] using formalin-fixed paraffin-embedded bone marrow tissues from MM patients. Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO, Glostrup, Denmark) for 15 min at room temperature. After washing, TdT, fluorescein isothiocyanate (FITC)-dUTP, and dATP (Boehringer Mannheim, Mannheim, Germany) were applied to the sections, which were then incubated in a moist chamber for 60 min at 37°C. Anti-FITC-conjugated antibody-peroxidase (POD converter, Boehringer Mannheim) was employed for detecting FITC-dUTP labeling, and color development was achieved with DAB containing 0.3% hydrogen peroxide solution. Sections were then observed under a microscope and the proportion of TUNEL-positive cells was determined by dividing the number of positively stained cells by the total cell number after counting more than 1,000 cells.

Immunohistochemistry for Survivin and Proliferative Cells

Four micrometer-thick sections of formalin-fixed paraffin-embedded bone marrow tissues from MM patients were cut on slides covered with adhesive. Sections were deparaffinized, and endogenous peroxidase was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Antibodies were then applied to identify survivin and to characterize proliferative cells. The primary antibodies included polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, San Antonio, TX) and monoclonal antibody Ki-67 (DAKO). All sections were developed using biotin-conjugated secondary antibodies against rabbit IgG or mouse IgG followed by a sensitive peroxidase-conjugated streptavidin system (DAKO) with DAB as the chromogen. Negative control staining procedure was performed using rabbit or mouse immunoglobulin of irrelevant specificity substituted with the primary antibody for each staining. The Ki-67-positive cell ratio was determined by dividing the number of positively stained cells by the total cell number after counting more than 1,000 cells.

Cell Lines

The establishment and characterization of the human MM tumor cell line RPMI8226 was previously described [25]. The cells were obtained from the American type culture collection (ATCC, Rockville, MD) and routinely maintained in RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Daiichi Seiyaku, Tokyo, Japan), 1% (v/v) penicillin at 100 units/ml (Invitrogen, Carlsbad, CA), and 1% (v/v) streptomycin at 100 units/ml (Invitrogen). The cells were grown in a humidified atmosphere at 37°C in 5% CO_2. We also generated the doxorubicin resistant variant of RPMI8226 cell line, designated DRR, according to a previously described method [25].

Statistical Analysis

Statistically significant differences in the quantitative analysis were determined using Wilcoxon's test for the comparison of paired MM samples before and after chemotherapy. Statistically significant differences for the quantitative analysis were determined using the Mann–Whitney's U test for comparison between control and MM samples, MM samples with good or poor prognosis, and the MM cell lines, RPMI8226 and DRR.

RESULTS

Expression of mRNA for MDR1 and LRP Determined by Real-Time Quantitative PCR

To quantitate the mRNA expression levels of MDR1 in MM bone marrow cells, real-time quantitative RT-PCR was performed using bone marrow samples from controls and MM samples before and after chemotherapy. As shown in Fig. 1A, the expression of MDR1 exhibited significant up-regulation in MM compared with the controls ($P < 0.01$). The overall expression in MM tended to increase after chemotherapy. More than half of the patients (14/26) exhibited up-regulated expression of MDR1 after chemotherapy in spite of the number of neoplastic cells possibly decreasing due to chemotherapy. Thus, MM cells in MDR1-up-regulated patients should express higher levels of MDR1 after chemotherapy. By contrast, 12 patients out of 26 with MM showed the reduced expression after treatment. This reflects the decrease in the number of MM cells in the bone marrow after chemotherapy.

Similarly, the expression of LRP exhibited significant up-regulation in MM compared with the controls ($P < 0.01$) (Fig. 1B). The expression of LRP tended to increase after chemotherapy. There were 17 patients with increased LRP expression, while

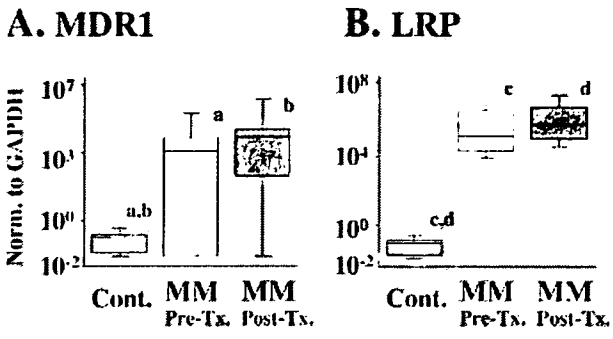

Fig. 1. Quantitative RT-PCR analysis of MDR1 (A) and LRP (B) in bone marrow samples from control cases (Cont.) ($n = 7$, white box) and MM patients before (Pre-Tx., light gray box) and after chemotherapy (Post-Tx., dark gray box) ($n = 26$). The relative intensity was calculated as [intensity of reaction of MDR1 (total Raji RNA, ng)]/[intensity of reaction of GAPDH (total Raji RNA, ng)]. The box plot graphs indicate the values for control and MM patients, where the bars indicate the 90 and 10% tile and the boxes indicate the 75–25% tile. Differences were significant between MDR1 expression in the controls and MM patients before chemotherapy ($^aP < 0.01$) and after chemotherapy ($^bP < 0.01$), LRP expression in the controls and MM patients before chemotherapy ($^cP < 0.01$), the controls and MM patients after chemotherapy ($^dP < 0.01$) seen by the Mann–Whitney's U-test.

decreased expression was observed in 9 after chemotherapy.

These results indicate that MDR1 and LRP are highly expressed in MM cells compared with control bone marrow cells and that this might be induced by chemotherapy in more than half of all patients. We thus focused on the analysis of patients with chemotherapy-induced up-regulation of MDR1 or LRP in the following study.

Expression of IAP Family Protein mRNA in MM Patients with Increased or Reduced Expression of MDR1 after Chemotherapy

Next, to quantitate the mRNA expression of IAP family proteins in MM bone marrow cells, real-time quantitative RT-PCR was performed using bone marrow samples from MM samples before and after chemotherapy. The values are indicated as the ratio postchemotherapy/prechemotherapy in Fig. 2. Among patients with increased expression of MDR1 after chemotherapy (14/26), the expression ratios for survivin, cIAP1, cIAP2, and XIAP were significantly higher in patients with poorer outcomes than in those with a good prognosis ($P < 0.05$, respectively) (Fig. 2A). In contrast, in patients with reduced MDR1 expression, no significant difference in the ratios of IAP expression was observed between patients with a good and poor prognosis (Fig. 2B).

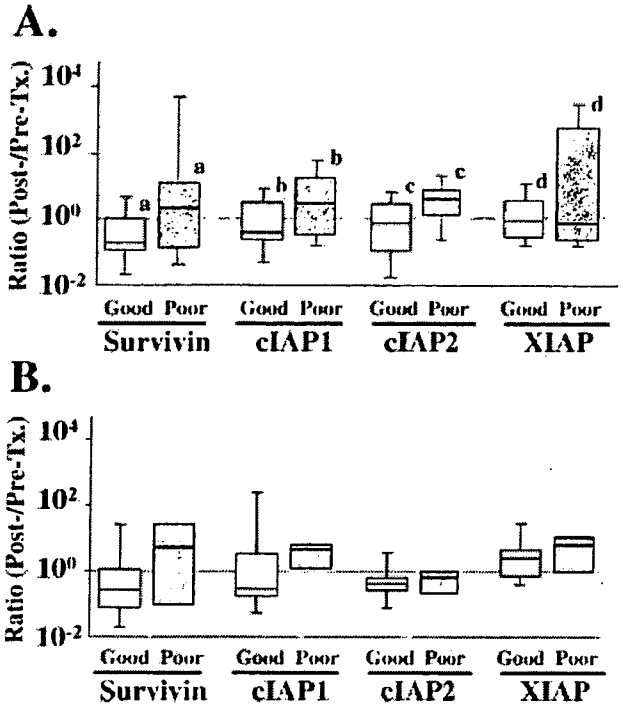

Fig. 2. Quantitative RT-PCR analysis of IAP family proteins in bone marrow samples from MM patients with increased (A, $n = 14$) or reduced (B, $n = 12$) expression of MDR1 after chemotherapy. The values are indicated as the ratio: [IAP expression after chemotherapy (Post-Tx.)/IAP expression before chemotherapy (Pre-Tx.)]. The box plot graphs compare the ratio of IAP expression between patients with a good prognosis (Good: white box) and those with a poor outcome (Poor: gray box). In MM patients with increased MDR1 expression (A), the ratios of expression of survivin, cIAP1, cIAP2, and XIAP were significantly higher in those with a poor outcome ($n = 7$) than in those with a good prognosis ($n = 7$) ($^{a-d}P < 0.05$, respectively, Mann–Whitney's U-test). In contrast, the expression levels of IAPs were not significantly different between patients with a good ($n = 3$) or poor prognosis ($n = 9$) in MM patients with reduced expression of MDR1 (B).

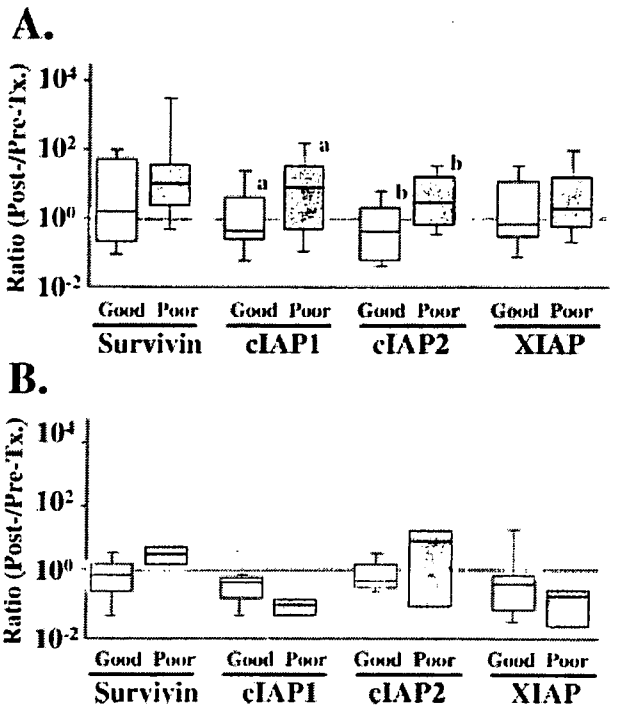

Fig. 3. Quantitative RT-PCR analysis of IAP family proteins in bone marrow samples from MM patients with increased (A, $n = 14$) or reduced (B, $n = 12$) expression of LRP after chemotherapy. In MM patients with increased LRP expression (A), the ratio of cIAP1 and cIAP2 expression was significantly higher in those with a poor outcome ($n = 9$) than in those with a good prognosis ($n = 8$) ($^{a,b}P < 0.05$, respectively, Mann–Whitney's U-test). In contrast, the ratios of expression of IAPs were not significantly different between patients with a good ($n = 2$) or poor prognosis ($n = 7$) in MM patients with reduced expression of LRP (B).

Thus, chemotherapy induced the overexpression of MDR1 and IAPs in patients with a poor outcome.

Expression of IAP Family Protein mRNA in MM Patients with Increased or Reduced Expression of LRP after Chemotherapy

To determine whether the chemotherapy-induced overexpression of LRP influenced the expression of IAP in association with the prognosis of the patients, the ratios for the expression of IAPs postchemotherapy/prechemotherapy were compared between patients with a good or poor prognosis. As shown in Fig. 3A, the ratios of cIAP1 and cIAP2 expression were significantly higher in patients with a poor outcome than in those with a good prognosis ($P < 0.05$, respectively). Similar to MDR1, patients with reduced expression of LRP exhibited no significant difference regarding the ratio between patients with a good and poor prognosis (Fig. 3B).

Immunolocalization of Survivin in MM Cells

To examine the histological localization of survivin in MM cells, immunohistochemical staining was performed in bone marrow samples from patients with survivin overexpression. As shown in Fig. 4A and B, survivin was localized to the nucleus of MM cells, although in a few cells cytoplasmic staining was also observed.

Apoptotic and Proliferative Cells in MM Samples before and after Chemotherapy

To determine the apoptotic and proliferative cell frequency of MM cells, the TUNEL-positive cell ratio and Ki-67 positive cell ratio were analyzed only for bone marrow samples in which foci of MM

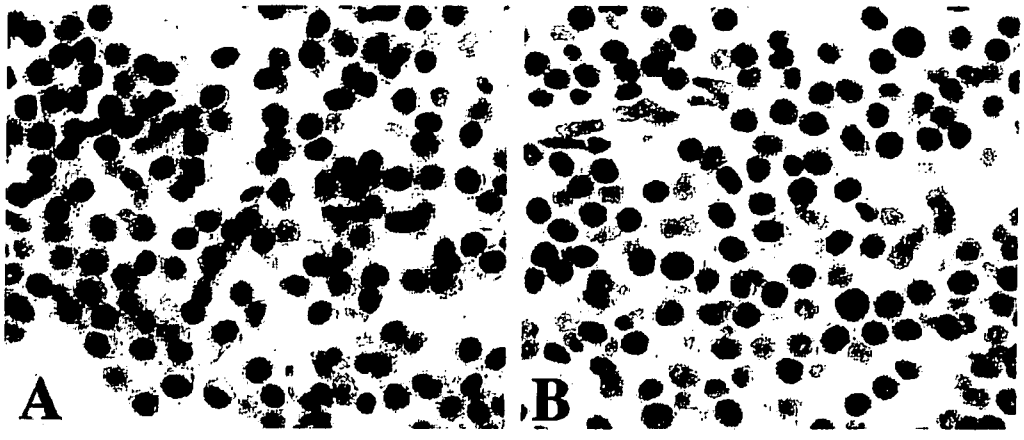

Fig. 4. Immunohistochemical localization of survivin in MM cells of the bone marrow before (A) and after chemotherapy (B). Note that the nuclei of more than half of the MM cells are positively stained although a few MM cells exhibit weak cytoplasmic staining. Differences in the ratio of survivin-positive MM cells were not remarkable before and after chemotherapy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells could be histologically identified. As summarized in Table I, the overall ratio of TUNEL-positive apoptotic cells exhibited a very mild increase after chemotherapy, although the difference was not significant. However, in cases with MDR1 up-regulation, the apoptotic cell ratio tended to decrease (pre-chemotherapy, median, 0.8; postchemotherapy, 0.4) after chemotherapy.

By contrast, the Ki-67-positive proliferative cell ratio significantly decreased due to chemotherapy ($P < 0.01$) (Table I). Differences were not significant between samples from MDR1 up-regulated patients and down-regulated patients as well as LRP up-regulated and down-regulated patients (data not shown). These results indicated that chemotherapy induced the reduction of proliferative MM cells and that the overexpression of MDR1 was associated with the reduction of apoptotic cells.

TABLE I. Apoptotic and Proliferative Cell Ratio of MM Cells of the Bone Marrow before and after Chemotherapy

Cells	Pre-Tx. ($n = 11$) (%)	Post-Tx. ($n = 12$) (%)
TUNEL$^+$	0.8 (0.0–6.0)[a]	1.4 (0.2–6.6)
Ki-67$^+$	48.4 (18.1–64.5)[b]	18.9 (10.0–23.5)[b]

[a]Values given in parentheses are median (Min.–Max.) values.
[b]The difference was significant between the Ki-67$^+$ cell ratios of MM samples before chemotherapy (Pre-Tx.) and after chemotherapy (Post-Tx.) ($P < 0.01$ by the Mann–Whitney's U-test).

Expression of IAP Family Protein mRNA in Human MM Cell Lines with Increased MDR1 and LRP Expression

Finally, to test whether MDR1 or LRP overexpression was correlated with the up-regulated expression of IAPs in the human MM cell line, IAP family protein mRNA expression was compared between the MM cell line RPMI8226 (8226) and doxorubicin-resistant variant (DRR) of RPMI8226. As shown in Fig. 5A,B, DRR cells expressed significantly higher levels of MDR1 and LRP ($P < 0.05$, respectively) than the original RPMI8226 cells. The DRR cells were also resistant to apoptosis induced by doxorubicin (data not shown). As expected from the findings using clinical samples from MM patients after chemotherapy, the DRR cells tended to express higher levels of IAPs than did the original RPMI8226 cells (Fig. 5C). The differences were significant between 8226 and DRR cells in terms of cIAP1, cIAP2, and XIAP expression ($P < 0.05$, respectively).

DISCUSSION

A strong correlation exists between MDR1 expression by tumor cells and previous chemotherapy in MM patients, and in particular is related to prior exposure to the natural agents vincristine and doxorubicin [26]. A in vitro study also reveals that exposure to doxorubicin selects for MDR1 expressing MM cell line cells [27]. We confirmed in the present study that the doxorubicin-resistant MM cell line, DRR, expressed higher levels of MDR1 as well as LRP than did the original RPMI8226 cells. The data from the clinical samples also supported these findings in the sense that the postchemotherapeutic induction of MDR1 as well as LRP overexpression was observed in more than half of the patients.

Regarding the prognosis, the MM patients who exhibited overexpression of MDR1 or LRP together with the up-regulation of IAPs exhibited a poor out-

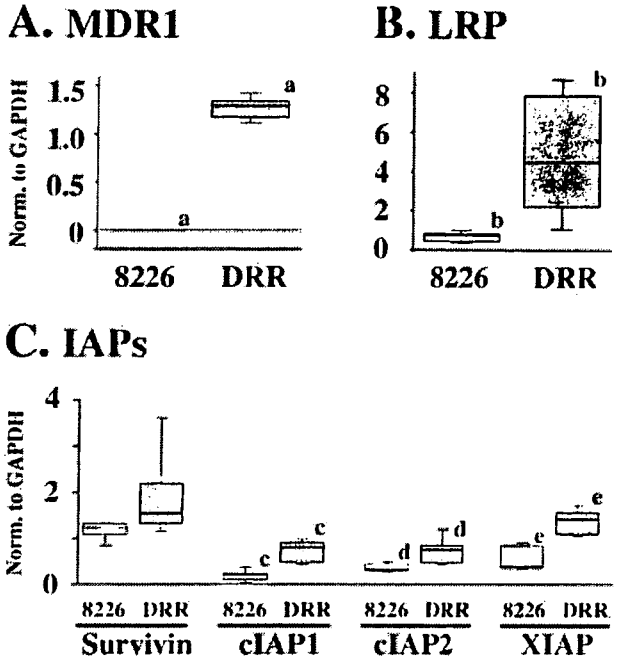

Fig. 5. Quantitative RT-PCR analysis of (A) MDR1, (B) LRP, and (C) IAPs in the MM cell line RPMI8226 (8226, white box) and doxorubicin-resistant variant RPMI8226 (DRR, gray box). As expected, the DRR cells expressed significantly higher levels of MDR1 (A) and LRP (B) than did the original RPMI8226 cells ($^{a,b}P < 0.05$, respectively, by Mann–Whitney's U-test). Note that the DRR cells tended to express higher levels of IAPs than did the original RPMI8226 cells (C). Differences were significant between cIAP1, cIAP2, and XIAP expression for 8226 and the DRR cells ($^{c-e}P < 0.05$, respectively, by Mann–Whitney's U-test).

come. It has been shown that the intracellular transport of IAP molecules is very important for the anti-apoptotic effects of these proteins [17]. Thus, the overexpression of MDR proteins mediates intracellular protein transport, might facilitate the transport of IAPs and introduce the hyper-function of these proteins in these MM patients. The apoptotic cell ratio was very low in the MM cells of these patients. Using the human HL60 leukemia cell line and its multidrug resistant line HL60R, Notarbartolo et al. [28] indicated that HL60R cells expressed much more MDR1 as well as survivin and cIAP2 than HL60. Treatment with doxorubicin strongly down-regulated survivin and XIAP in HL60 cells, and in contrast, the levels of these IAP mRNA were much less affected by the treatments in HL60R cells. Our data using the MM cell line also revealed that the DRR cells expressed higher levels of IAPs as well as MDRs than did the RPMI8226 cells. Western blot analysis confirmed that the expression dynamics of IAPs at the protein level was almost parallel with the dynamics at the mRNA level in MM (RPMI8226) cells after treatment with anti-cancer drug, although the levels of protein expression changed a little slowly (manuscript in preparation). These results suggest that IAPs might play a key role in tumor resistance to chemotherapeutic drugs in association with MDRs, although the interrelationships between IAPs and MDR gene products are far from being completely understood.

It would be also important to clarify the mechanisms responsible for the up-regulation of IAP family proteins in MM cells after chemotherapy. Insulin-like growth factor-1 (IGF-1) and interleukin-6 (IL-6) promote the proliferation of MM cells. IGF-1 stimulates the sustained activation of NF-κB and Akt and up-regulates a series of intracellular anti-apoptotic proteins including FLIP, survivin, cIAP-2, and XIAP. In contrast, IL-6 does not cause sustained NF-κB activation, induces less pronounced Akt activation, and increases the expression of only survivin [29]. We previously revealed that TNF-α is present locally in the bone marrow microenvironment and is associated with the regulation of cellular proliferation/apoptosis in hematological diseases [30]. TNF-α induces NF-κB nuclear translocation, cIAP-1 and cIAP-2 up-regulation, and proliferation in MM cells [31]. Thus, the expression of IAP is controlled by complex cellular signals. Further study is necessary to elucidate the mechanism of specific IAP induction in MM cells during chemotherapy by clarifying the genome-wide expression profiles of apoptosis-associated molecules using microarray technique.

Concerning the apoptotic process of MM cells, it has been shown that murine and human MM cell lines are sensitive to TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis [32]. Adriamycin sensitized this type of apoptosis in an adriamycin-resistant MM cell line [33]. The apoptotic signaling was dependent on a mitochondrial apoptotic pathway but not on a death receptor-mediated apoptosis pathway including that for the IAP family proteins cIAP-1, cIAP-2, and XIAP. By contrast, coexposure of human leukemia/MM cells to TRAIL and the cyclin-dependent kinase inhibitor flavopiridol (FP) increases mitochondrial injury and apoptosis [34]. TRAIL/FP induced no discernible changes in survivin expression, a modest decline in the level of cIAP, and resulted in the marked transcriptional down-regulation of XIAP. Thus, TRAIL/FP-induced apoptosis in human leukemia/MM cells disturbs XIAP-associated anti-apoptotic processes.

In conclusion, we showed that chemotherapy induced up-regulation of the expression of IAP mRNA and significantly worsened the prognosis of MM patients who exhibited chemotherapy-induced overexpression of MDRs. These results suggest that MDRs and IAPs might cooperate or interact to make MM

cells more resistant to chemotherapy, although analysis using a larger group of patients should be performed. In vitro data using RPMI8226 and DRR cell lines also suggested this association. Further studies using knocking down system of protein expression should clarify the mechanism responsible for MDRs as well as IAPs induction in MM cells under chemotherapy and a novel chemotherapeutic strategy should be considered for blocking IAPs in MDR-overexpressing MM cells.

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Expression dynamics of drug resistance genes, multidrug resistance 1 (MDR1) and lung resistance protein (LRP) during the evolution of overt leukemia in myelodysplastic syndromes

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Abstract

It is well-known that leukemic cells of overt leukemia (OL) that have transformed from myelodysplastic syndromes (MDS) are more resistant to chemotherapy as compared with de novo AML cells. Thus, to examine the expression levels of drug-resistant genes and their alterations with the development of OL in MDS, the expression of mRNA for MDR1 and LRP was determined in bone marrow samples from control, de novo AML, MDS, MDS at the time of OL transformation (MDS → OL), and after transformation (OL) by quantitative real-time RT-PCR. The expression of MDR1 in MDS bone marrow at the time of initial diagnosis was as low as that for control subjects. However, the expression level was significantly elevated at the time of the development of OL (MDS → OL) compared with the initial MDS subjects ($P < 0.05$), while expression was relatively reduced after OL development (OL). The expression of LRP was significantly higher in MDS and MDS → OL samples than control subjects. However, the high expression of LRP in MDS → OL was significantly reduced after OL development (OL). The expression levels of drug-resistant genes in MDS → OL or OL were not significantly higher than those of de novo AML samples, although LRP expression in MDS or MDS → OL was relatively higher than that of de novo AML. Detecting increases in the expression of MDR1 would be useful for predicting OL development in MDS patients.

Keywords: MDR1; LRP; MDS; Overt leukemia; Bone marrow

Introduction

Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis with frequent apoptosis (Fontenay-Roupie et al., 1999; Gupta et al., 1999; Harris et al., 1999; Heaney and Golde, 1999; Kitagawa et al., 1997, 1998, 1999; Parcharidou et al., 1999; Sawanobori et al., 2003; Vardiman et al., 2002) and a high risk of progression to overt leukemia (OL) of acute myeloid leukemia (AML) type (Harris et al., 1999; Heaney and Golde, 1999; Kitagawa et al., 1989). Patients with OL following MDS have a low response rate to conventional chemotherapy compared with de novo AML patients. This could be due in part to a higher incidence of drug resistance gene expression in MDS than in de novo AML (Leith et al., 1997; Lepelley et al., 1994; Marie et al., 1991; Poulain et al., 2000; Wattel et al., 1998) and anti-apoptotic mechanisms present in $CD34^+$ cells of MDS when OL developed (Suárez et al., 2004).

In recent years, important advances have been achieved in the treatment of patients with AML. However, AML in the elderly continues to result in a dismal outcome (Nabhan et al., 2005; Pinto et al., 2001). It has been suggested that blast cells from elderly AML patients frequently show unfavorable karyotypes in association with high expression of multidrug resistance 1 (MDR1) (Del Poeta et al., 1999; Leith et al., 1997; Nabhan et al., 2005; Suárez et al., 2005) as well as increased resistance to apoptosis (Cascavilla et al., 2000; Van Stijn et al., 2003). MDS is also a disease that mainly affects the elderly. Thus, to focus on the contribution of drug-resistant genes to the

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chemotherapy-resistant nature of OL that has transformed from MDS, we collected bone marrow samples from MDS cases in the present study. In these cases, we could follow over the course of development of OL. Using quantitative RT-PCR, we traced the expression of MDR proteins during the course of OL development from MDS. Among the MDR proteins, MDR1, lung resistance protein (LRP) and multidrug-resistance-associated protein (MRP1), expression level of MRP1 did not correlate with the prognosis of MDS patients, while the expression levels of MDR1 as well as LRP did (Poulain et al., 2000). Therefore, we examined the expression of MDR1 and LRP.

The level of expression of MDR1 was relatively high in bone marrow cells from de novo AML samples compared with control bone marrow. However, the level of expression in MDS at the time of initial diagnosis was similar with that of control samples. At the time of OL evolution, the expression level showed a remarkable increase followed by a relative decrease thereafter. By contrast, the expression of LRP in MDS at the time of initial diagnosis was higher than that of control subjects and exhibited a slight increase at the time of OL evolution (MDS → OL). Then, it was significantly reduced after OL development (OL). The significance of expression dynamics of drug-resistant genes in the bone marrow cells of MDS to OL is discussed.

Materials and methods

Patients

Fresh frozen bone marrow samples from controls (7 cases, age, median 55, max. 74, min. 43; male:female, 1:6), MDS (7 patients, 3 with RA and 4 with RAEB; male:female, 5:2; age, median 68, max. 77, min. 55) who developed overt leukemia later in the course (duration, 3 months to 3 years) and de novo AML (12 patients of M2 by FAB classification, male:female, 5:7; age, median 62, max. 76, min. 49) patients were analyzed. To rule out the influence of aging on bone marrow cells, age-matched control cases were analyzed. Diagnoses were based on standard clinical and laboratory criteria, including cell morphology (Harris et al., 1999; Heaney and Golde, 1999; Vardiman et al., 2002). All samples were collected at the time of the initial aspiration biopsy, and the samples from de novo AML contained more than 80% blasts. The patients were not infected with specific viruses including HTLV-1 and had not been treated prior to the study. In MDS, we followed the bone marrow changes just before and after the transformation to OL and further chronological changes determined at more than three points were analyzed. During the course of OL development, patients were not treated with the specific anti-tumor chemotherapy. We determined the beginning of blast proliferation in MDS patients and followed the changes in the number of blasts in the bone marrow. MDS samples were collected at the time of initial diagnosis. Furthermore, MDS → OL samples were collected from bone marrow containing 20–30% blasts and OL samples were collected from bone marrow with blasts comprising beyond 30% of all bone marrow cells.

The procedures followed were in accord with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

Preparation of RNA and quantitative assay for MDR1 and LRP using TaqMan RT-PCR

RNA was extracted from frozen bone marrow samples of control subjects with no hematological disorders, de novo AML patients and MDS patients using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Although the proportions of cell types were variable in the bone marrow samples of MDS, we examined the overall expression of MDR genes by total bone marrow cells because of the clonal nature of this disease entity.

For quantitative RT-PCR, fluorescent hybridization probes and the TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with the ABI Prism 7900HT Sequence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for MDR1, LRP, and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes that were used were as follows. The sequence of the forward primer for MDR1 mRNA was 5′-GTCTACAGTTCGTAATGCTGACGT-3′ and that of the reverse primer was 5′-TGTGATCCACGGACACTCCTAC-3′; the TaqMan probe was 5′-CGCTGGTTTCGATGATGGAGTCATTG-3′. For LRP mRNA, the forward primer was 5′-CGCTGTGATTGGAAGCACCTA-3′ and the reverse primer was 5′-CGGGAGGCAGCTCTTTCTC-3′; the TaqMan probe was 5′-ATGCT-GACCCAGGACGAAGTCCT-3′. The forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGAGT-3′ and the reverse primer was 5′-GAAGATGGTGATGGGATTTC-3′; the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. Conditions for one-step RT-PCR were as follows: 2 min at 50°C (Stage 1, reverse transcription), 10 min at 95°C (Stage 2, RT inactivation and AmpliTaq Gold activation) and then 45 cycles of amplification for 15 s at 95°C and 1 min at 60°C (Stage 3, PCR). The expression of MDR1 and LRP was quantified according to a method described elsewhere (Yamamoto et al., 2004). Briefly, the intensity of the reaction was evaluated from the quantity of total RNA in Raji cells (ng) corresponding to the initial number of PCR cycles to reveal the linear increase in reaction intensity (threshold cycle) for each sample on a logarithmic standard curve. Data on the quantity of RNA (ng) for the MDR1 and LRP were normalized using that for GAPDH in each sample.

Statistical analysis

Statistically significant differences in the quantitative analysis were determined using the Mann–Whitney's *U* test for comparisons between control, de novo AML, MDS, MDS → OL, and OL samples except for the comparison of paired samples from MDS, MDS → OL, and OL, for which Wilcoxon's test was used.

Results

Expression of mRNA for MDR1 determined by real-time quantitative PCR

To quantitate the mRNA expression levels of the MDR1 in MDS bone marrow cells, real-time quantitative RT-PCR was performed using bone marrow from control, de novo AML, MDS, MDS → OL, and OL samples. As shown in Fig. 1, the expression of MDR1 exhibited relative up-regulation in de novo AML compared with the controls. By contrast, the mRNA for MDR1 revealed similar levels of expression in MDS bone marrow and control marrow. At the time of OL development (MDS → OL), the MDR1 expression level became as high as that of de novo AML. The difference was significant between MDS and MDS → OL ($P < 0.05$). However, expression was slightly reduced after OL development (OL). Furthermore, we followed more precise dynamics for a couple of cases with MDS. Fig. 2 shows the expression intensity of MDR1 during the course and the proportion of blasts in the bone marrow at each point. As indicated, the MDR1 expression transiently increased when the blast population increased to circa 20%, and then expression was reduced in spite of the blast numbers still increasing in the bone marrow.

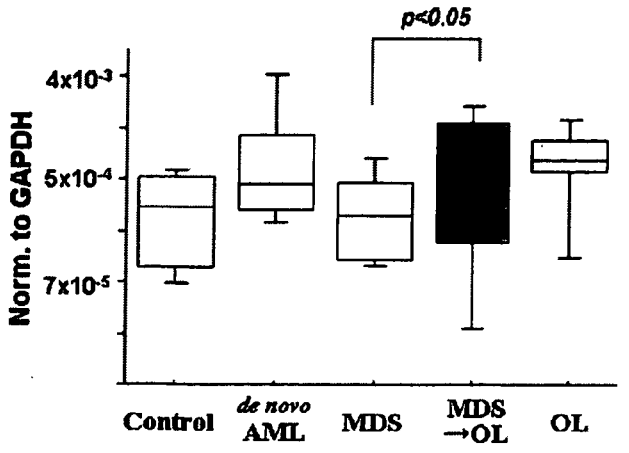

Fig. 1. Quantitative RT-PCR analysis of MDR1 in control ($n = 7$), de novo AML ($n = 12$), MDS ($n = 7$) at the time of initial diagnosis, at the time of OL development (MDS → OL), and after OL evolution (OL). The relative intensity was calculated as (intensity of reaction of MDR1 [total Raji RNA, ng])/(intensity of reaction of GAPDH [total Raji RNA, ng]). The box plot graphs indicate the values for AML and MDS cases, where the bars indicate the 90th and 10th percentile and the boxes indicate the 75th to 25th percentile. The difference was significant between MDS and MDS → OL ($^aP < 0.05$) by Wilcoxon's test.

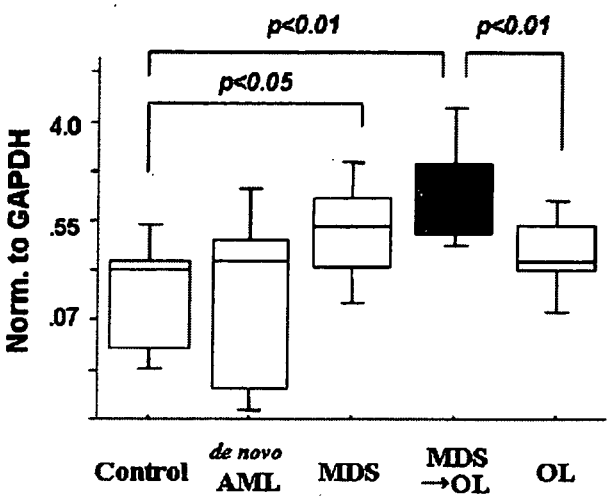

Fig. 3. Quantitative RT-PCR analysis of LRP in control ($n = 7$), de novo AML ($n = 12$), MDS ($n = 7$), MDS → OL, and OL samples. Differences were significant between the samples using Mann–Whitney's U test or Wilcoxon's test: control and MDS ($^aP < 0.05$), control and MDS → OL ($^bP < 0.01$), and MDS → OL and OL ($^cP < 0.01$).

These results indicate that a high expression of MDR1 might not be characteristic of OL transformed from MDS because the level of expression was similar with that of de novo AML. However, a significant difference in the MDR1 expression in MDS and MDS → OL is a useful finding for predicting the evolution of OL in MDS.

Expression of mRNA for LRP determined by real-time quantitative PCR

Next, to quantitate the mRNA expression dynamics of LRP in MDS bone marrow cells, real-time quantitative RT-PCR was performed using bone marrow from control, de novo AML, MDS, MDS → OL, and OL samples. As shown in Fig. 3, the expression of LRP exhibited a significant up-regulation in MDS and MDS → OL samples compared with the controls. mRNA expression was significantly decreased after transformation to OL from MDS → OL samples. However, differences were not remarkable between de novo AML and MDS, AML and MDS → OL, or AML and OL. These results indicate that the expression dynamics of LRP during the course of OL development in MDS were not significant. However, expression was reduced after transformation to OL. Detailed dynamics on two cases are shown in Fig. 4. Similar to MDR1, LRP expression also revealed a transient peak at the time when the blast population increased and gradually decreased thereafter. However, the period for the peak expression of LRP was relatively earlier than that of MDR1 in both cases.

In any event, the expression dynamics of LRP did not explain the chemotherapy-resistant nature of MDS → OL or OL bone marrow compared with de novo AML because the increased expression of LRP was transient in MDS during the

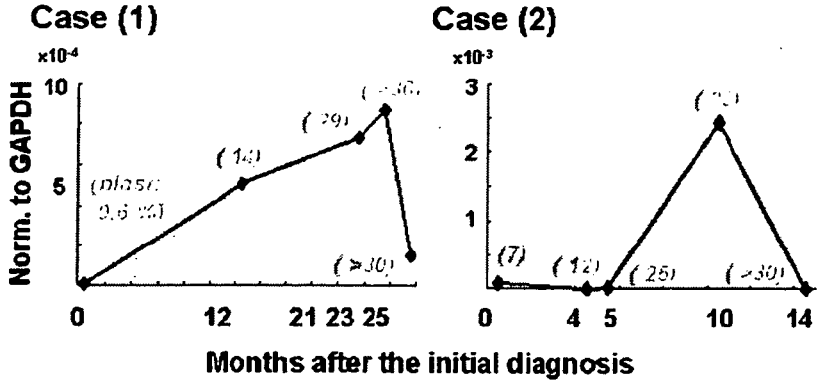

Fig. 2. Dynamics of the expression of mRNA for MDR1 in two cases of MDS during the course of OL development. Although the expression in seven cases was determined chronologically, representative two cases are shown. Case 1 was a 76-year-old male who was initially diagnosed with RA and Case 2 was a 55-year-old female who was initially diagnosed with RAEB. The percentage of blasts in the bone marrow is indicated at each point. Note the peak expression of MDR1 23 months after the initial diagnosis for Case 1 and 10 months for Case 2 when the evolution of OL occurred. The blasts accounted for 20 to 30% of all bone marrow cells.

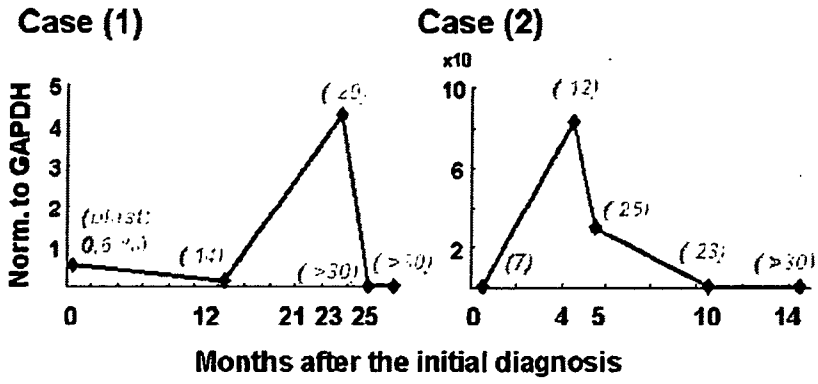

Fig. 4. Dynamics of the expression of LRP mRNA in two cases of MDS during the course of OL development. The representative cases are the same as those indicated in Fig. 2. Note that the peaks for the expression of LRP 21 months after the initial diagnosis for Case 1 and 4 months for Case 2 were relatively earlier than those of MDR1 in both cases.

course of OL development and expression in OL was not significantly higher than that of de novo AML.

Discussion

Regarding the expression of MDR1 in myeloid neoplasms, previous studies revealed that over-expression occurred in de novo AML and MDS samples (Leith et al., 1999; Lepelley et al., 1994). Expression was more frequent in AML in the elderly than AML in younger people (Leith et al., 1999) and in "high risk" MDS than in "low risk" MDS (Lepelley et al., 1994). Similarly, LRP over-expression is more frequent in MDS than in de novo AML (Lepelley et al., 1998). However, little is known about the quantitative changes in the expression intensity of mRNA for these genes in particular patients. In other words, the expression dynamics of MDR1 and LRP have not been previously studied in association with the pathogenesis of MDS, MDS → OL, and OL.

The expression of MDR1 is associated with a CD34-positive stem cell phenotype in MDS (List et al., 1991; Sonneveld et al., 1993) as well as in de novo AML (Guerci et al., 1995). Therefore, in MDS, the number of CD34-positive immature cells in the bone marrow strongly influences the expression intensity of MDR1. Our data which show that MDR1 expression increased at the time of OL development (MDS → OL) might reflect the increase in immature cells in the bone marrow. However, the reduced expression after OL development (OL) could not be explained by the number of CD34-positive cells because it was still increasing after OL development.

LRP over-expression is more frequent in MDS than in de novo AML and, as in AML, is only partially correlated with MDR1 expression (Lepelley et al., 1998). In the present study, the expression of LRP in MDS or MDS → OL was stronger than that in control samples, but the expression in de novo AML was similar with that of control samples. Although the number of samples was inadequate to evaluate rather a heterogeneous entity of hematological malignancy, MDS, we found a significant elevation in expression in MDS samples even at the time of initial diagnosis. However, LRP is not a prognostic factor for response to chemotherapy and survival in MDS (Lepelley et al., 1998). Our findings were consistent with this observation in the sense that the MDS → OL or OL samples did not exhibit higher expression than the initial MDS samples.

The decline in MDR1 and LRP protein expression after the evolution of OL may be attributable to gene silencing, for example, by DNA hypermethylation. Although this interpretation is speculative, over-expression of MDR1 and LRP in MDS and MDS → OL would be a reversible change while the down-regulation in OL samples might be irreversible/reversible.

Recently, modulation of drug resistance transporters has been considered as a strategy for treating myelodysplastic syndromes (Ross, 2004). Several drugs capable of modulating and decreasing MDR1, such as quinine, tamoxifen, calcium channel blockers, and cyclosporine A, have been used for treating poor-risk AML (Advani et al., 1999; List et al., 1993; Solary et al., 1992; Tallman et al., 1999). Furthermore, a potent inhibitor of the MDR1 efflux pump, valspodar, is used for patients with relapsed or refractory AML and high-risk MDS with mitoxantrone, etoposide, and cytarabine (phase III trial) (Greenberg et al., 2004). The present data suggest that this type of chemotherapy is attractive for treating patients when OL occurs in MDS. Although our data indicate that patients with OL after transformation from MDS exhibit low level expression of MDR1, these drugs might be effective for preventing drug-induced up-regulation of MDR1 expression.

In conclusion, we investigated the transient over-expression of MDR1 mRNA during the progression from MDS to OL, although the expression was relatively decreased after transformation was completed. Clinically, it would be useful to detect increases in the expression of MDR1 mRNA for predicting the evolution of OL in MDS patients. Although the levels of mRNA do not directly reflect protein expression status, further study is warranted to clarify the mechanisms of up- and down-regulation of MDR1/LRP expression in MDS, MDS → OL, and OL in association with the chemotherapy-resistant nature of this disease.

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GENE EXPRESSION PROFILING OF RAT LIVER TREATED WITH SERUM TRIGLYCERIDE-DECREASING COMPOUNDS

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ABSTRACT — We have constructed a large-scale transcriptome database of rat liver treated with various drugs. In an effort to identify a biomarker for interpretation of plasma triglyceride (TG) decrease, we extracted 218 probe sets of rat hepatic genes from data of 15 drugs that decreased the plasma TG level but differentially affected food consumption. Pathway and gene ontology analysis revealed that the genes belong to amino acid metabolism, lipid metabolism and xenobiotics metabolism. Principal component analysis (PCA) showed that 12 out of 15 compounds were separated in the direction of PC1, and these 12 were separated in the direction of PC2, according to their hepatic gene expression profiles. It was found that genes with either large or small eigenvector values in principal component PC 2 were those reported to be regulated by peroxisome proliferator-activated receptor (PPAR)α or constitutive androstane receptor (CAR), respectively. In fact, WY-14,643, clofibrate, gemfibrozil and benzbromarone, reported to be PPARα activators, distributed to the former, whereas propylthiouracil, omeprazole, phenobarbital, thioacetamide, methapyrilene, sulfasalazine and coumarin did to the latter. We conclude that these identified 218 probe sets could be a useful source of biomarkers for classification of plasma TG decrease, based on the mechanisms involving PPARα and CAR.

KEY WORDS: Triglyceride, Liver, CAR, PPAR, Toxicogenomics

INTRODUCTION

The toxicogenomics project was a 5-year collaborative project by the National Institute of Biomedical Innovation (NIBIO), the National Institute of Health Science (NIHS), and 15 pharmaceutical companies in Japan that started in 2002 (Urushidani and Nagao, 2005). Its aim was to construct a large-scale toxicology database of transcriptome for prediction of toxicity of new chemical entities in the early stage of drug development. About 150 chemicals, mainly medicinal compounds, were selected, and gene expression in liver (also kidney in some cases) was comprehensively analyzed by using Affymetrix GeneChip®. In 2007, the project was finished and the whole system, consisting of the database, the analyzing system and the prediction system, was completed and named as TG-GATEs (Genomics Assisted Toxicity Evaluation System developed by Toxicogenomics Project, Japan).

In toxicity studies, plasma triglyceride (TG) decrease is often observed. Because plasma TG level can be influenced mostly by nutritional status, decrease in food consumption is one of the factors for its change. For activator of peroxisome proliferator-activated

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receptor (PPAR)α, it is a hot field of drug development, and this drug facilitates the expression of genes related to fatty acid β–oxidation (Schoonjans *et al.*, 1996), subsequently lowering plasma TG level. Phenobarbital (PB), an antiepileptic barbiturate derivative, decreases plasma TG level but increases plasma total cholesterol without decrease in food consumption in rats (Kiyosawa *et al.*, 2004; Hall *et al.*, 1990). Kiyosawa *et al.*(2004) proposed a mechanism of serum cholesterol elevation via up-regulation of hepatic cholesterol synthesis. So far, PB is not reported to be a PPARα activator, and the mechanism of plasma TG decrease by PB is not well understood. In these cases, plasma TG decrease could be a target of the drug in one case, or a sign of toxicity in another case, each with different mechanisms. Thus, identification of the mechanisms behind plasma TG decrease during drug treatment would enable both seed discovery and interpretation of toxicity.

One of the main purposes of TG-GATEs is to identify biomarkers for toxicity evaluation. Although there have been various reports describing strategies to extract marker genes from the transcriptome data (Hibbs *et al.*, 2004; Mutlib *et al.*, 2006; Tan *et al.*, 2006), the best way has not been established. In the present study, we have started to identify candidates of potential biomarker genes for interpretation of the fundamental mechanism(s) of plasma TG decrease, since our database contains several drugs that cause plasma TG decrease.

MATERIALS AND METHODS

Animals and treatments

Male Crj:CD(SD)IGS rats were purchased from Charles River Japan Inc., (Kanagawa, Japan) at 5-weeks of age. After a 7-day quarantine and acclimatization period, the animals were divided into groups of 5 animals using a computerized stratified random grouping method based on the body weight for each age. The animals were individually housed in stainless-steel cages in a room that was lighted for 12 hr (7:00-19:00) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21-25°C with a relative humidity of 40-70%. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, Oriental Yeast Co., Japan). Rats in each group were orally administered with various drugs suspended or dissolved either in 0.5% methylcellulose solution (MC) or corn oil according to their dispersibility. At the time when the present analysis was performed, 15 compounds in our database were found to decrease the plasma triglyceride level during repeated administration (Table 1). Of these, isoniazid (INAH, 50, 100, 200 mg/kg; MC), phenobarbital (PB, 10, 30, 100 mg/kg; MC), thioacetamide (TAA, 4.5, 15, 45 mg/kg; MC), benzbromarone (BBr, 20, 60, 200 mg/kg; MC), methapyrilene (MP, 10, 30, 100 mg/kg: MC), amiodarone hydrochloride (AM, 20, 60, 200 mg/kg; MC), gemfibrozil (GFZ, 30, 100, 300 mg/kg; corn oil) and sulfasalazine (SS, 100, 300, 1000 mg/kg; MC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Alpha-naphthylisothiocyanate (ANIT, 1.5, 5, 15 mg/kg; corn oil) was purchased from Kanto Chemical (Tokyo, Japan). Coumarin (CMA, 15, 50, 150 mg/kg; corn oil), propylthiouracil (PTU, 10, 30, 100 mg/kg; MC) and WY-14,643 (WY, 10, 30, 100 mg/kg; corn oil) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Carbon tetrachloride (CCL4, 30, 100, 300 mg/kg; corn oil), clofibrate (CFB, 30, 100, 300 mg/kg; corn oil) and omeprazole (OPZ, 100, 300, 1000 mg/kg; MC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Body weights were recorded every day while food consumption was recorded every 4 days during repeated dosing and expressed as g/day. The animals were treated for 3, 7, 14 or 28 days, and they were sacrificed 24 hr after the last dosing. Blood samples were collected to heparinized tube under ether anesthesia from the abdominal aorta after which the animals were sacrificed.

Blood chemistry analysis

Heparinized blood samples were centrifuged at 1,600 × g for 20 min to obtain plasma and the concentration of TG was determined using an automated clinical analyzer (Japan Bioassay Research Center and Anpyo Center; HITACHI 7070, Hitachi Ltd., Food and Drug Safety Center: COBAS MIRA plus, Roche Diagnostics, Bozo Research Center: TBA-120FR, Toshiba Lab Medical, Tokyo, Japan).

Microarray analysis

After collecting the blood, the animals were euthanized by exsanguinations from abdominal aorta under ether anesthesia. An aliquot of the sample (about 30 mg) for RNA analysis was obtained from the left lateral lobe of the liver in each animal immediately after sacrifice, kept in RNAlater®(Ambion, Austin, TX, USA) overnight at 4°C, and frozen at −80°C until use. Liver samples were homogenized with the buffer RLT supplied in RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and total RNA was isolated according to the

Table 1. List of compounds used in the present study.

Compound (abbreviation)	No. of mobilized genes (ANOVA, $p<0.05$)	Usage	Hepatotoxicity	Proposed toxicological action
alpha-naphthylisothiocyanate (ANIT)	1063	hepatotoxins	cholestasis	toxic metabolite can injure epithelial cells in biliary duct
amiodarone hydrochloride (AM)	640	antiarrhythmic, antianginal agents	phospholipidosis	oxidative stress by free radical
benzbromarone (BBr)	1720	uricosuric agents	hepatomegaly	peroxisome proliferators-like action
carbon tetrachloride (CCL4)	2262	hepatotoxins	hepatocellular carcinoma fatty liver	production of toxic metabolite that leads to peroxidative injury of membrane lipids and membrane perturbation
clofibrate (CFB)	1800	antilipemic agents	hypertrophy, hepatocellular carcinoma	peroxisome proliferator
coumarin (CMA)	1005	hepatotoxins	necrosis	formation of coumarin 3,4-epoxide
gemfibrozil (GFZ)	1943	antilipemic agents	hepatomegaly	peroxisome proliferator
isoniazid (INAH)	1482	antituberculous agents	necrosis	generation of a reactive metabolite from acetylhydrazine
methapyrilene (MP)	4910	antiallergic, hypnotic, sedative agents	carcinoma	induction of hepatic cell proliferation, lipid peroxidation of the liver
omeprazole (OPZ)	1441	antiulcer agents	elevation of serum enzyme	unknown
Phenobarbital (PB)	548	hypnotics, sedatives, anticonvulsants	hepatocellular tumor	induction of c-fos gene expression
propylthiouracil (PTU)	1544	antithyroid agents	elevation of serum enzyme	thyroxin synthesis inhibitor
sulfasalazine (SS)	860	antiinflammatory, antirheumatic, antiinfective agents	genotoxicity, carcinogenicity	antiinflammatoric, antibacterial actions, inhibition of production of cytokines
thioacetamide (TAA)	5712	hepatotoxins	carcinogenicity	hepatocarcinogen
Wy-14,643 (WY)	4307	hepatotoxins	carcinoma hepatomegaly	peroxisome proliferator

manufacturer's instructions. Microarray analysis was conducted on 3 out of 5 samples for each group by using GeneChip® RAE 230A probe arrays (Affymetrix, Santa Clara, CA, USA), containing 15,923 probe sets. The procedure was conducted basically according to the manufacturer's instructions using Superscript Choice System (Invitrogen, Carlsbad, CA, USA) and T7-(dT)24-oligonucleotide primer (Affymetrix) for cDNA synthesis, cDNA Cleanup Module (Affymetrix) for purification, and BioArray High yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA) for synthesis of biotin-labeled cRNA. Ten micrograms of fragmented cRNA was hybridized to a RAE230A probe array for 18 hr at 45°C at 60 rpm, after which the array was washed and stained by streptavidin-phycoerythrin using Fluidics Station 400 (Affymetrix) and scanned by Gene Array Scanner (Affymetrix). The digital image files were processed by Affymetrix Microarray Suite version 5.0. Microarray image data were analyzed with GeneChip Operating Software (Affymetrix).

Statistical analysis

Plasma TG and food consumption results were expressed as means ± SD. They were analyzed by Bartlett test that evaluates the homogeneity of variance. If the variances were homogenous, ANOVA was applied. If the variances were heterogeneous, Kruskal-Wallis test was performed. When ANOVA resulted in a statistical difference between the groups, Dunnett test was applied. When Kruskal-Wallis test resulted in statistically different groups, Dunnett type mean rank test was performed. Identification of genes related to plasma TG decrease and gene expression data were analyzed by Welch ANOVA for the dose level and applied with Benjamini and Hochberg False Discovery Rate as a multiple-testing correction. In these tests, a significant level at $p<0.05$ was considered acceptable (Snedecor and Cochran, 1989).

GeneChip data were normalized by the global median normalization method using GeneSpring version 7 (Agilent Technologies Inc., Palo Alto, CA, USA). Probe sets with present or marginal call in at least 1 of 48 samples (N=3 for 4 time points and 4 dose levels) were selected. Principal component analysis (PCA) of the GeneChip data was performed using Spotfire DecisionSite ver. 8.2 (Spotfire, Somerville, MA, USA).

Pathway and Gene Ontology (GO) analysis

The identified probe sets were subjected to analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and GO analysis by DAVID (Database for Annotation, Visualization, and Integrated Discovery; http://apps1.niaid.nih.gov/david/) using Fisher's exact test (Dennis *et al.*, 2003). Level 5 analysis was adopted.

RESULTS

Plasma biochemistry and food consumption

Rats were treated with each compound (ANIT, AM, BBr, CCL4, CFB, CMA, GFZ, INAH, MP, OPZ, PB, PTU, SS, TAA and WY) by gavage for up to 4 weeks. The TG concentration and food consumption results are shown in Fig. 1. All compounds that we selected showed a TG-decreasing property during the dosing period, while their effects on food consumption differed. In BBr-, CMA-, OPZ- and SS-treated animals, food consumption transiently dropped in the first 3 days while it returned to normal thereafter. In AM-, INAH- and TAA-treated rats, food consumption was depressed throughout the dosing period. PTU-treatment decreased food consumption from day 15 whereas MP-treatment decreased at day 29. ANIT-, CFB-, CCL4, GFZ-, PB-, and WY-treatment affected the food intake only slightly.

Identification of genes related to the plasma TG-decreasing property

After filtering the probe sets (with present or marginal call in at least 1 of 48 samples), Welch ANOVA with multiple testing correction was applied to each compound to extract significantly mobilized probe sets. As shown in Table 1, the numbers of extracted probe sets varied among the compounds, from 640 (AM) to 5,712 (TAA). We then selected the probe sets that were commonly changed from more than 10 out of 15 compounds, and 218 probe sets were obtained. KEGG pathway analysis revealed that pathways related to "proteasome", "fatty acid metabolism", "amino acid metabolism", and "bile acid biosynthesis" were mainly altered in liver treated with these compounds (Table 2). GO analysis showed that some groups (other than cellular lipid metabolism) related to xenobiotics metabolism, such as carboxylic acid metabolism, and glucuronosyltransferase and aldehyde dehdrogenase activity, were also affected in liver (Table 3).

Principal component analysis (PCA)

To assess the expression profiles of identified

probe sets, PCA with 218 probe sets for data of all 15 compounds were performed. As shown in Fig. 2, each sample was separated from control according to the expression of these probe sets. Each sample tended to have a smaller value in the component PC 1 as the treatment period increased, and each was distinctly separated into either direction in component PC 2. It should be noted that liver treated with WY, BBr, GFZ and CFB had a relatively large principal component PC 2, while PTU, OPZ, PB, TAA, MP, SS and CMA showed small PC 2. Liver treated with AM for 28 days had a near zero value in PC 2. Some compounds such as ANIT, CCL4 and INAH did not change their position very much.

To elucidate which genes contributed more for each principal component, eigenvector values of each probe sets were examined. As shown in Table 4, "vanin 1", "similar to Aig1 protein", "CD36 antigen", and "cell death-inducing DNA fragmentation factor, alpha subunit-like effecter A" had large eigenvector values for PC 2. Meanwhile, "glutathione *S*-transferase A5, aldehyde dehydrogenase family 1, member A1", "liver UDP-glucuronosyltransferase, phenobarbital-inducible form", "carbonic anhydrase 2" and "cytochrome P450, family 2, subfamily b, polypeptide 15" had small values for PC 2 (Table 5). "Aldehyde dehydrogenase family 1, member A1", "glutathione *S*-transferase A5", "vanin 1", "carboxylesterase 2 (intestine, liver)" and "CD36 antigen" had smaller eigenvector values for PC 1 (Table 6).

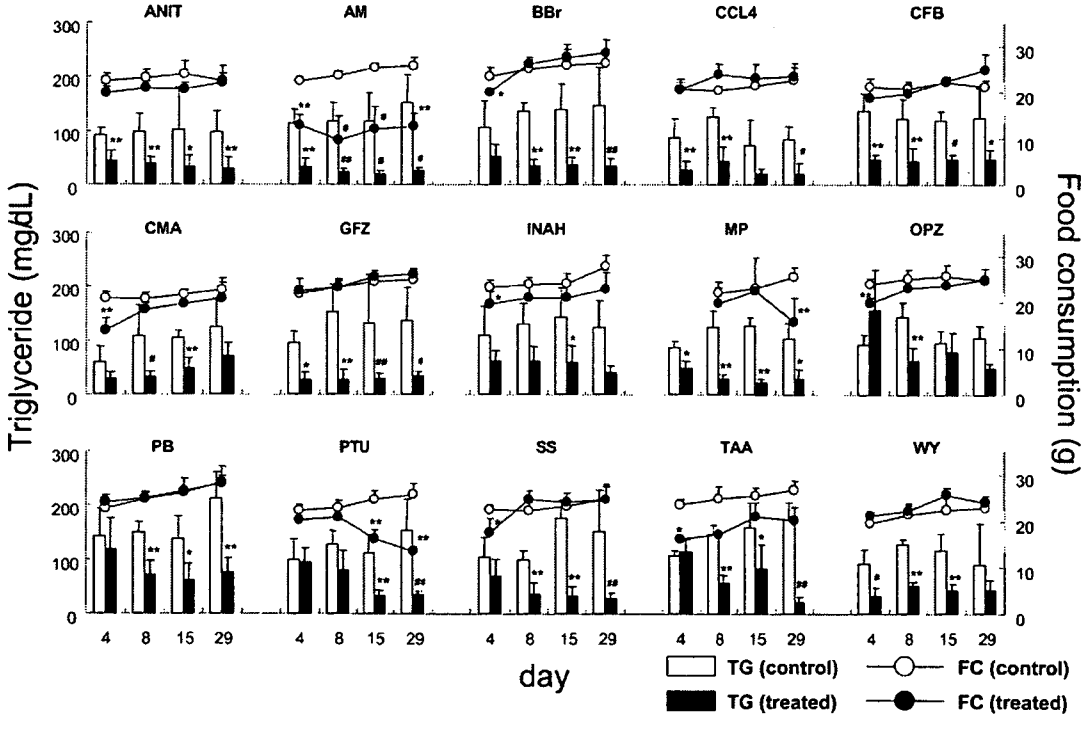

Fig. 1. Effects of TG-decreasing compounds on food consumption and plasma TG level.
Six-week-old male Sprague-Dawley rats were gavaged with each compound for 3, 7, 14 or 28 days, and they were sacrificed 24 hr after the last dosing. Food consumption was recorded every 4 days during dosing and blood samples were collected at sacrifice. Plasma TG concentrations were estimated as described in materials and methods. For simplicity, the data of the highest dose were presented for each compound. Open (control) and filled (treated) columns represent plasma TG concentration. Open (control) and filled (treated) circles represent food consumption. Values are expressed as mean ± SD of 5 rats each for each time and compound. Significant difference from control rat: (*p<0.05, **p<0.01: Dunnett test, #p<0.05, ##p<0.01: Dunnett type mean rank test). MP-food consumption on day 4 was not determined.

As PC 2 was considered to be indirectly related to cholesterol metabolism (see DISCUSSION), three compounds with the smallest values for both PC 1 and PC 2, i.e., PB, OPZ and PTU were selected, and their effects on plasma cholesterol level are shown in Fig. 3. It is obvious from the figure that OPZ and PTU, which had smaller PC 2 values than PB, significantly increased plasma cholesterol.

DISCUSSION

Lowering of the plasma TG level is often observed in rat toxicity studies. It would be useful to elucidate its mechanism not only for safety evaluation of drugs but also for finding seeds of lipid-lowering agents. In the course of our trials to identify useful toxicity biomarkers from our large-scale database, we selected plasma TG decrease as a toxicological phenotype, and picked up 15 such compounds from 40 (the number of compounds available at the time when the

Table 2. KEGG pathways of the identified 218 probe sets related to plasma TG.

Term	Count	p value
proteasome	8	1.66E-06
fatty acid metabolism	10	4.61E-06
tryptophan metabolism	9	3.44E-05
bile acid biosynthesis	4	0.00997
histidine metabolism	4	0.00997
propanoate metabolism	4	0.0224
fatty acid biosynthesis (path 2)	3	0.0236
pyruvate metabolism	4	0.0289
valine, leucine and isoleucine degradation	4	0.0289
nitrogen metabolism	3	0.0364
arginine and proline metabolism	4	0.0492

Statistically extracted 218 probe sets related to plasma TG were categorized by KEGG pathway. The terms with significantly high counts (Fisher's exact test; $p<0.05$, calculated by DAVID: http://apps1.niaid.nih.gov/david/) are presented in the table.

Table 3. Gene ontology of the identified 218 probe sets related to plasma TG.

Category	Term	Counts	p value
Biological process	carboxylic acid metabolism	15	5.25E-06
	electron transport	14	1.83E-04
	fatty acid metabolism	8	6.04E-04
	response to chemical substance	7	0.00164
	cellular lipid metabolism	10	0.0115
	amino acid metabolism	6	0.0154
	protein catabolism	11	0.0492
Molecular function	glucuronosyltransferase activity	6	5.01E-05
	aldehyde dehydrogenase activity	3	0.0249
Cellular component	endoplasmic reticulum	15	1.25E-05
	microsome	9	1.93E-04
	micorobody	5	0.00726
	peroxisome	5	0.00726
	proteasome complex (SENSU EUKARYOTA)	4	0.00829
	proteasome core complex (SENSU EUKARYOTA)	4	0.00829
	mitochondrion	15	0.0103

Statistically extracted 218 probe sets related to plasma TG were categorized by gene ontology. The terms with significantly high counts (Fisher's exact test; $p<0.05$, calculated by DAVID: http://apps1.niaid.nih.gov/david/) are presented in the table.

present analysis was performed). Since our database has a broad variety of compounds for hepatic toxicity, each compound has different properties for drug efficacy and toxicity (Table 1).

As shown in Fig. 1, the effects of these compounds on food consumption were not similar. It is well known that plasma TG levels depend largely upon nutrition, and the results suggested that there should have been multiple factors other than a simple general toxicity. To clarify the multiple mechanisms in plasma TG homeostasis, we extracted 218 probe sets as commonly changed genes in more than 10 out of 15 compounds (commonly changed in two-thirds of the compounds). The fact that there were no probe sets commonly changed in all the compounds (data not shown) also suggested that there are multiple factors involved in the mechanism of plasma TG decrease.

KEGG pathway analysis suggested that the proteasome-, fatty acid metabolism-, tryptophan metabolism-, bile acid biosynthesis-, and histidine metabolism-related pathways were involved (Table 2). Since TG is an important source of energy, it is understood

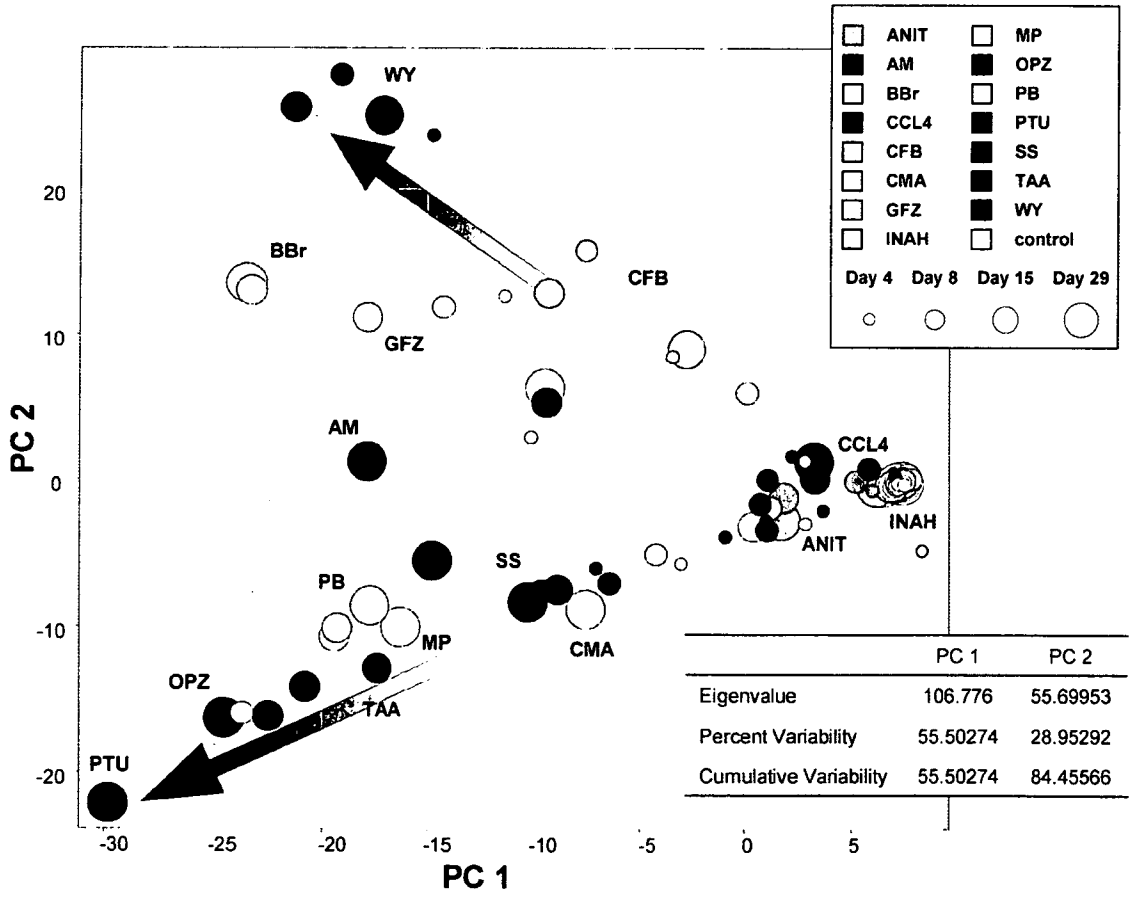

Fig. 2. Principal component analysis of gene expression profiles.
PCA of gene expression profiles was performed using identified 218 probe sets for the data of the highest dose of 15 compounds at various time points. The selection of the 218 probe sets related to plasma TG decrease is described in materials and methods. The values used in the analysis were the normalized signal values. Each spot represents the mean of the normalized gene expression value. The abbreviation for each drug is summarized in Table 1. The eigenvalue, percent variability, and cumulative variability for PC 1 and PC 2 are shown in the table on the lower right corner.

that pathways related to energy homeostasis such as fatty acid synthesis and amino acid metabolism were affected. GO analysis showed that the carboxylic acid metabolism- and glucuronosyltransferase activity-related genes were also affected by TG-decreasing compounds (Table 3). UDP-glucuronosyltransferase, one of the extracted genes, is of major importance in conjugation and subsequent elimination of potentially toxic xenobiotics (Bock *et al.*, 1990). PB and OPZ, which lowered plasma TG level in the present study, were previously reported to have the ability to induce this enzyme (Bock *et al.*, 1990; Masubuchi *et al.*, 1997). These results indicated that the xenobiotics metabolism pathway might have a role in plasma TG decrease.

To examine the basis of the gene expression profile of each sample, we performed PCA on hepatic gene expression profiles of the 15 compounds. In PCA, three clear clusters were identified (Fig. 2). All compounds except ANIT, CCL4 and INAH were uniformly dispersed into smaller PC 1 with either large or small PC 2, i.e., WY, BBr, GFZ and CFB were dispersed into small PC 1 with large PC 2, whereas PTU, OPZ, PB,MP, TAA, SS and CMA into small PC 1 with small PC 2. AM was exceptionally found in the middle position of PC 2.

In PC 2, "vanin 1", "similar to Aig1 protein", "CD36 antigen", "cell death-inducing DNA fragmentation factor, and alpha subunit-like effecter A (CIDEA)" had larger eigenvector values (Table 4), meaning that these genes made a great contribution to increasing PC 2 values in each liver sample. Vanin 1 is

Table 4. Top five probe sets with the largest eigenvector values for second principal component in the PCA shown in Fig. 2.

Ranking	Probe ID	PC 2	Description
1	1389253_at	0.610	Vanin 1 (predicted)
2	1375845_at	0.291	Similar to Aig1 protein
3	1367689_a_at	0.230	CD36 antigen
4	1389179_at	0.180	Cell death-inducing DNA fragmentation factor, alpha subunit-like effecter A (predicted)
5	1386901_at	0.153	CD36 antigen

Each eigenvector value was calculated by Spotfire DecisionSite.

Table 5. Top five probe sets with the smallest eigenvector values for second principal component in the PCA shown in Fig. 2.

Ranking	Probe ID	PC 2	Description
1	1371089_at	−0.526	Glutathione *S*-transferase A5
2	1387022_at	−0.187	Aldehyde dehydrogenase family 1, member A1
3	1370698_at	−0.113	Liver UDP-glucuronosyltransferase, phenobarbital-inducible form
4	1386922_at	−0.0742	Carbonic anhydrase 2
5	1371076_at	−0.0586	Cytochrome P450, family 2, subfamily b, polypeptide 15

Each eigenvector value was calculated by Spotfire DecisionSite.

Table 6. Top five probe sets with the smallest eigenvector values for first principal component in the PCA shown in Fig. 2.

Ranking	Probe ID	PC 1	Description
1	1387022_at	−0.630	Aldehyde dehydrogenase family 1, member A1
2	1371089_at	−0.491	Glutathione *S*-transferase A5
3	1389253_at	−0.370	Vanin 1 (predicted)
4	1368905_at	−0.163	Carboxylesterase 2 (intestine, liver)
5	1367689_a_at	−0.162	CD36 antigen

Each eigenvector value was calculated by Spotfire DecisionSite.