Evaluation of the drug sensitivity and expression of 16 drug resistance-related genes in canine histiocytic sarcoma cell lines

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Running head: CANINE HISTIOCYTIC SARCOMA
Canine histiocytic sarcoma (HS) is an aggressive tumor type originating from histiocytic cell lineages. This disease is characterized by poor response to chemotherapy and short survival time. Therefore, it is of critical importance to identify and develop effective antitumor drugs against HS. The objectives of this study were to examine the drug sensitivities of 10 antitumor drugs. Using a real-time RT-PCR system, the mRNA expression levels of 16 genes related to drug resistance in 4 canine HS cell lines established from dogs with disseminated HS were determined and compared to 2 canine lymphoma cell lines (B-cell and T-cell). These 4 canine HS cell lines showed sensitivities toward microtubule inhibitors (vincristine, vinblastine and paclitaxel), comparable to those in the canine B-cell lymphoma cell line. Moreover, it was shown that P-gp in the HS cell lines used in this study did not have enough function to efflux its substrate. Sensitivities to melphalan, nimustine, methotrexate, cytarabine, doxorubicin and etoposide were lower in the 4 HS cell lines than in the 2 canine lymphoma cell lines. The data obtained in this study using cultured cell lines could prove helpful in the developing of advanced and effective chemotherapies for treating dogs that are suffering from HS.

**KEY WORDS:** dog, drug resistance, histiocytic sarcoma, microtubule inhibitors, TP53 gene
Canine histiocytic sarcoma (HS) is a rare tumor type originating from histiocytic cell lineages, including dendritic cells (DCs) and macrophages [2, 26], and are characterized by aggressive biological behavior and poor prognosis. Canine HS is subdivided into two categories: solitary HS and disseminated HS. In addition, hemophagocytic HS has recently been described as a different subtype of canine HS that originates from macrophages. Monotherapy with lomustine (CCNU) is often the treatment of choice for HS [34], and there have been several reports describing its response to chemotherapy with doxorubicin [39], liposomal entrapped doxorubicin [39] and paclitaxel [28]. Nevertheless, HS often acquires multidrug resistance to these antineoplastic agents within a short time, leading to a median survival time of less than 100 days [30, 34, 35]. Therefore, there is a pressing need to develop effective antitumor drugs against HS and identify the factors that lead to the chemoresistance. Although the drug sensitivities of HS cells against some of the chemotherapeutic agents have been examined in vitro [14, 17, 36, 42], so far there have been no comprehensive studies on the drug sensitivity/resistance against a series of antineoplastic agents of various categories in canine HS cell lines.

To date, many studies have been carried out in order to elucidate drug resistance in various human tumors in humans, and a number of genes related to drug resistance have been uncovered. Over-expression of ATP-binding cassette transporters (ABC transporters) including \textit{ABCB1} [27] and \textit{ABCC1} [27], \textit{ABCG2} [6] is known to be the major mechanism underlying reduced drug accumulation in tumor cells. Mutant \textit{TP53} and over-expression of \textit{Bcl-2} lead to decreased apoptosis [5, 21]. GSTA4 and GSTP1,
members of the Glutathione S-transferase (GST) family, are known to induce
detoxification of cytotoxic drugs [7, 18]. Moreover, DNA repair pathways are also
known to be involved in the development and acquisition of drug resistance.
Furthermore, previous reports suggested that O⁶-alkylguanine DNA alkyltransferase,
which is encoded by the O⁶-methylguanine DNA methyltransferase (MGMT) gene, is
associated with resistance to alkylating agents [4, 13, 19]. Loss of DNA mismatch repair
(MMR) genes is also known to lead to drug resistance [1, 10, 11].

In veterinary medicine, the expression of P-gp is enhanced in tumor cells that
were obtained from dogs with relapsed lymphoma and chemotherapy-resistant
lymphoma [22, 24]. Our recent study revealed that the epigenetic regulation of ABCB1
was associated with sensitivity to vincristine in canine lymphoma cell lines [37].
Inhibition of survivin increased sensitivities to CCNU and doxorubicin, and also
influences the biological behavior of canine HS cell lines [42]. However, no studies
have yet been able to adequately account for the underlying cause of the short survival
time even after chemotherapy treatment in dogs afflicted with HS.

The objectives of this study were to evaluate the sensitivities of HS cell lines
toward a series of antineoplastic agents and investigate the associations between this
sensitivity and the expression levels of drug resistance-related genes in canine HS cell
lines.

MATERIALS AND METHODS

Cell lines and cell culture: Four canine HS cell lines (CHS-1, CHS-2, CHS-3 and
DH82) and 2 lymphoma cell lines (Ema and CLBL-1) were used in this study. CHS-1,
CHS-2 and CHS-3 [3] were established from the tumor tissues of dogs with HS. None of the 3 dogs from which these HS cell lines were established had received any chemotherapy. DH82 [41] was established from a dog with malignant histiocytosis. Ema [15] was established from a dog with abdominal T-cell lymphoma showing drug resistance, whereas CLBL-1 [32] was established from a dog with diffused large B-cell lymphoma (DLBCL) that had not been subjected to chemotherapy. CLBL-1 was used as a representative canine B-cell lymphoma cell line sensitive to vincristine, whereas Ema was used as a representative canine T-cell lymphoma cell line that is resistant to vincristine [37]. Our previous study revealed that due to epigenetic regulation, the expression level of ABCB1 was much higher in Ema than in the CLBL-1 cell line [37].

HS cell lines were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Biowest, Nuaille, France) in a humidified atmosphere containing 5% CO₂. The 2 lymphoma cell lines were cultured at 37°C in RPMI-1640 culture medium containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂.

Preparation and storage of antitumor drugs: Ten antitumor drugs were used in this study, melphalan (Sigma-Aldrich, St. Louis, MO, USA), nimustine hydrochloride (Tokyo Chemical Industry, Tokyo, Japan), methotrexate (Tokyo Chemical Industry), cytarabine (Wako, Tokyo, Japan), vincristine sulfate (Wako), vinblastine sulfate (Wako), paclitaxel (Wako), doxorubicin hydrochloride (Wako), etoposide (Wako) and Paccal Vet®️, water-soluble micellar paclitaxel (WSMP). WSMP was kindly provided by Oasmia Pharmaceutical AB and the Nippon Zenyaku Kogyo Co., Ltd. (ZENOAQ). The stock solutions of all drugs were prepared according to the manufacturer’s instructions.
and stored at -20°C. Working concentrations of the chemotherapeutic agents were prepared by diluting stock solutions with saline.

**Drug sensitivity assay:** The 50% inhibitory concentration (IC$_{50}$) values for each drug were determined for the 6 cell lines. After pre-incubation for 24 hr, cells (5×10$^4$ cells/mL) were co-cultured in a 96-well plate with various concentration of each drug or saline for 48 hr. After cultivation, cell viability was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer’s instruction. The absorbance was measured using a Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA), and IC$_{50}$ values for the 6 cell lines were determined. All samples were examined in three independent experiments.

**Quantitative analysis of mRNA by real-time RT-PCR:** Expression levels of mRNAs for ABCB1, ABCC1, ABCG2, LRP, TP53, p21$^{\text{waf1}}$, Bcl-2, survivin, GSTA4, GSTP1, MGMT, MSH2, MSH3, MSH6, MLH1 and PMS2 were evaluated using a real-time RT-PCR system. MSH2, MSH3, MSH6, MLH1 and PMS2 are genes involved in DNA mismatch repair [23]. Primer sequences for these genes related to drug resistance were designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and are listed in “Supplementary file 1”. Primers for surviving and p21$^{\text{waf1}}$ were prepared as described previously [38, 42]. For normalization purposes, HMBS and TBP were selected as appropriate internal controls. Detailed information of the real-time RT-PCR procedure is shown in “Supplementary file 2”.

**Examination of TP53 mutations:** To examine mutations in the coding regions of the TP53 gene in the 6 cell lines, genomic DNA sample was extracted from each cell line using the QIAamp® DNA Blood Mini Kit (QIAGEN, Limburg, Netherlands). The
sequence of the *TP53* genomic DNA was divided into seven fragments, and seven pairs of primers were selected based on previous report [9]. Primer sequences are listed in “Supplementary file 3”. The DNA samples were amplified by PCR using AmpliTag Gold® 360 (Applied Biosystems, Foster City, CA, U.S.A.), and primer pairs were constructed according to the manufacturer’s instructions for each fragment as well as *MMP3*, which is located on the same chromosome as *TP53*. Amplification of products was confirmed by electrophoresis. The PCR products were inserted into a T/A cloning vector (pGEM-T Easy) (Promega Corporation, Leiden, The Netherlands) and subjected to sequence analysis. The sequence of each fragment was analyzed using the BigDye terminator v3.1/1.1 Cycle Sequencing Kit (Applied Biosystems) and the Applied Biosystems 3130XL genetic analyzer (Applied Biosystems). At least five clones from each sample were sequenced.

*Nucleotide sequencing of *ABCB1* cDNA:* The sequence of entire cDNA of *ABCB1* gene was divided into eight fragments (fragments 1 to 8), and eight primer pairs were designed (Supplementary file 4). Sequences of the primers were based on the GenBank database (AF269224). Nucleotide sequences of the amplified fragments were analyzed directly from the PCR products using the BigDye terminator v3.1/1.1 Cycle Sequencing Kit (Applied Biosystems) and the Applied Biosystems 3130XL genetic analyzer (Applied Biosystems). The results were confirmed by two independent experiments.

*Western blot analysis for P-gp:* Ten µg of protein extracted from each cell line was separated by SDS-PAGE and blotted onto a PVDF membrane. The membranes were blocked in 5% skimmed milk and incubated with primary antibodies against P-gp.
(murine monoclonal: C219, Merck Millipore, Darmstadt, Germany) diluted at 1:100 for 12 hr at 4 °C, or β-actin (murine monoclonal: AC-15, Sigma-Aldrich) diluted at 1:5,000 for 1 hr at room temperature. Then, the membranes were incubated with HRP-labeled goat anti-mouse IgG antibodies (1:2,000; Santa Cruz Biotechnology, Dallas, TX, U.S.A) for 1 hr at room temperature. After incubation, positive immunoreactivity was detected using Luminata Forte Western HRP Substrate (Merck Millipore) and visualized using a ChemiDoc XRS Plus (Bio-Rad Laboratories).

**Rhodamine 123 efflux test:** Function of P-gp was evaluated by testing its efflux function for its substrate dye as reported previously. One million cells of each cell line were incubated with 200 ng/ml of Rhodamine 123 (Sigma-Aldrich) in medium at 37°C for 15 min. After washing in medium, the cells were incubated in Rhodamine 123-free medium at 37°C for 1 hr, either with or without 2 µM of cyclosporine (Sigma-Aldrich). After incubation, the cells were washed in medium and subjected to flow cytometric analysis using the FACSCalibur (BD Biosciences, Franklin Lakes, NJ, U.S.A). Cells that had not been exposed to Rhodamine 123 were used as negative controls. The results were confirmed by two independent experiments.

**Statistical analysis:** Data were expressed as mean ± standard deviation (SD). One-way ANOVA followed by Tukey’s post-hoc test was performed for multiple comparisons of relative mRNA quantities using the STATMATE (ATMS, Tokyo, Japan) software. *P*-values of less than 0.05 were considered statistically significant.

**RESULTS**

**Drug sensitivity assay:** The IC₅₀ values for the 6 cell lines are shown in Table 1.
and Fig. 1. For alkylating agents (i.e., melphalan and nimustine hydrochloride), the 4 HS cell lines showed higher IC$_{50}$ values than the 2 lymphoma cell lines. Similarly, the HS cell lines showed higher IC$_{50}$ values for antimetabolites (methotrexate and cytarabine), doxorubicin and etoposide compared to the lymphoma cell lines. Meanwhile, the HS cell lines demonstrated similar or lower IC$_{50}$ values for microtubule inhibitors (vincristine sulfate, vinblastine sulfate, paclitaxel and WSMP) compared to the 2 lymphoma cell lines. The IC$_{50}$ values for vincristine sulfate in the HS cell lines were similar to that in CLBL-1, a canine B-cell lymphoma cell line reported to be sensitive to vincristine [37]. For vinblastine and paclitaxel, HS cell lines showed lower IC$_{50}$ values than CLBL-1.

Quantitative analysis of mRNA by real-time RT-PCR: The expression levels of 16 drug resistance genes are shown in “Supplementary file 5”. In addition, comparisons of 5 genes showed that the relative quantities were significantly different between HS, and lymphoma cell lines are illustrated in the graphs (Fig. 2). Among the drug efflux transporters (ABCB1, ABCC1, ABCG2 and LRP), the expression levels of ABCB1 in 3 HS cell lines (CHS-1, CHS-2 and DH82) were significantly higher than those in CLBL-1. Furthermore, the expression level of ABCB1 was significantly higher in Ema. The expression levels of ABCG2 in the 4 HS cell lines were significantly higher, compared to those in the 2 lymphoma cell lines. However, there were no significant differences observed in the expression levels of ABCC1 and LRP between HS cell lines and lymphoma cell lines.

The 4 HS cell lines exhibited significantly lower expression levels of TP53 than the 2 lymphoma cell lines. The expression levels of $p21^{\text{waf1}}$ were significantly higher in
the 4 HS cell lines than in the 2 lymphoma cell lines. No significant differences were observed in the levels of survivin and Bcl-2 between the HS and lymphoma cell lines. With regard to the DNA repair genes (MSH2, MSH3, MLH1, PMS2 and MGMT), the expression levels of MSH6 in the 4 HS cell lines were significantly lower than in the 2 lymphoma cell lines. However, there were no significant differences between the HS and lymphoma cell lines with respect to the other 4 genes.

Mutation of the TP53 gene: Of the 6 cell lines, mutations in the TP53 gene were identified in 3 HS cell lines and 1 lymphoma cell line (Fig. 3). CHS-1 harbored a point mutation in exon 10, resulting in the introduction of a stop codon at codon 326. CHS-2 harbored 3 nucleotide deletions in exon 6 at codon 206 (Val). In these cell lines, no clone representing the wild-type TP53 sequence was detected in the five clones examined. No gene amplification could be observed by PCR using 7 primer pairs complementary to the canine TP53 gene in the DH82 cell line, although MMP3 was amplified normally. CLBL-1 had heterozygous point mutations at exon5 (Ala\textsuperscript{125} to Val), but no mutations were observed in the CHS-3 and Ema cell lines.

Nucleotide sequencing of the ABCB1 gene: In the sequence analysis, no mutation was found in the cDNAs of ABCB1 gene of 6 cell lines. The results were compared to the coding sequence of canine ABCB1 gene (GenBank association number AF269224).

Western blot for P-gp: In Western blotting, a distinct band of approximately 170 kD corresponding to P-gp was detected in Ema (Fig.5). In CHS-1 and DH82, a faint corresponding band was found. Expression of P-gp was not detected in CHS-3 and CLBL-1.

Rhodamine 123 efflux test: In the Rhodamine 123 efflux test, efflux of the dye
was observed in Ema, whereas the dye was retained in other cell lines (Fig. 4).

**DISCUSSION**

In the 4 HS cell lines examined in this study, the IC\textsubscript{50} values of vincristine were 1.77 to 2.69 ng/ml, which were similar to or less than that in a vincristine-sensitive canine B-cell lymphoma cell line (CLBL-1) (1.86 ng/ml), but much lower than that in a vincristine-resistant canine T-cell lymphoma cell line (Ema) (52.4 ng/ml). Similar results were obtained for the IC\textsubscript{50} values of vinblastine, paclitaxel and WSMP, indicating that the 4 HS cell lines demonstrated sensitivities toward the tested microtubule inhibitors, comparable to the chemosensitive canine B-cell lymphoma cell line. Currently, there are only a few published studies available that examined the pharmacokinetics of antitumor drugs in dogs; however, the C\textsubscript{2min} value of vincristine was reportedly 39.8 ± 9.18 ng/ml in 5 beagle dogs when intravenously injected at a dose of 0.07 mg/kg [44]. Moreover, the C\textsubscript{max} value of WSMP was recently reported as 14 ± 6.6 µg/ml in dogs when it is intravenously administered at a dose of 150 mg/m\textsuperscript{2} [40], and a complete or partial response was observed in 59% of dogs with mastocytoma when injected intravenously at a median dose of 145 (range, 135 -150) mg/m\textsuperscript{2} [31]. The suggested dose of vincristine is 0.7 mg/m\textsuperscript{2} in representative chemotherapeutic protocol for canine lymphoma [12, 25], and the recommended dose of paclitaxel is 132 mg/m\textsuperscript{2} [29]. Therefore, these results suggest that the IC\textsubscript{50} values of the microtubule inhibitors that were used in this study can be achieved at a clinical dosage level, and that these drugs may be good candidates for the effective treatment of canine HS. However, no reports have thus far described the efficacy of monotherapy with microtubule inhibitors.
for the treatment of dogs with HS. Consequently, further studies would be required to evaluate the efficacies of these candidate drugs in treatment applications for canine HS.

The 4 HS cell lines showed relatively high IC₅₀ values for alkylating agents (melphalan and nimustine hydrochloride), antimetabolites (methotrexate and cytarabine), antitumor antibiotic (doxorubicin hydrochloride) and topoisomerase inhibitor (etoposide) in comparison to CLBL-1. Although there have been several reports on the treatment of canine HS with CCNU and nimustine hydrochloride (ACNU) [35], from their response rates (46% and 50%, respectively) and medium survival times (59 and 48 days, respectively), efficacy of these agents does not seem to be satisfactory. Moreover, the number of dog patients evaluated for the efficacy of ACNU was small (6 dogs), and 2 of the 3 responders did not achieve complete remission. Canine HS cell lines examined in this study were generally resistant to ACNU; therefore, further effort is necessary to examine the clinical efficacy of other chemotherapeutic agents in dog patients suffered from HS.

Here, it was shown that the expression levels of *ABCB1* in 3 HS cell lines (CHS-1, CHS-2 and DH82) were significantly higher than that in CLBL-1, though significantly lower than that in Ema. It was previously reported that the product of *ABCB1*, P-gp, was involved in the resistance to vincristine, vinblastine and paclitaxel [8, 16]. However, the IC₅₀ values of these 3 drugs were similar or lower in HS cell lines, compared to CLBL-1. In Western blot, the band corresponding to P-gp was not found in CHS-2 and CHS-3 and very faint in CHS-1 and DH82. Moreover, no efflux of the substrate dye was observed in the 4 HS cell lines in the Rhodamine 123 efflux test, and no mutation of the *ABCB1* gene was identified in the 4 HS cell lines. These results indicated that the 4
canine HS cell lines did not express P-gp with sufficient function, although 3 of them had measurable amount of ABCBI mRNA. It was conceivable that translation of the mRNA to P-gp was impaired for some reasons.

The expression levels of TP53 in the 4 HS cell lines were very low in comparison to the 2 lymphoma cell lines. Especially, in the DH82 cell line, nearly no TP53 mRNA was detected by the quantitative PCR analysis. Therefore, down regulation of TP53 might be associated with resistance to a wide range of antitumor drugs possibly via a mechanism that circumvents apoptosis. Moreover, 2 out of the 4 HS cell lines harbored mutations in the TP53 gene, and it was shown that in these mutations, there were no wild-type alleles present. Result obtained from sequencing of TP53 also indicated that the coding region of TP53 was in fact lost in the DH82 cell line. These results implied that aberrations in the TP53 gene might be a common genetic alteration in canine HS. A further study is needed to know the frequency of the aberration of TP53 gene in the primary tumor tissues obtained from dogs that developed HS. Moreover, direct experiment of transferring wild-type TP53 gene into TP53-deficient canine HS cell line will reveal its influence on the drug resistance in the neoplastic cells of canine HS.

Since the expression of p21WAF1 is induced by P53, its expression level is usually reduced in cells with inactivated p53. However, the expression levels of p21WAF1 were significantly higher in the HS cell lines than in the lymphoma cell lines (Supplementary file. 5). Although wild-type p53 promotes the expression of p21WAF1 [20], p53-independent induction of p21 expression has been observed in several cell types including human breast cancer cells and the canine MDCK cell line [33, 43]. Therefore, it is suggested that the expression of p21WAF1 in the HS cell lines examined in this study
may have been induced by p53-independent mechanisms.

In this study, we screened sensitivities demonstrated by canine HS cell lines to conventional antitumor drugs in comparison to canine lymphoma cell lines. The 4 canine HS cell lines examined have shown high IC$_{50}$ values of alkylating agents, antimetabolites, doxorubicin and etoposide, whereas they exhibited relatively low IC$_{50}$ values of microtubule inhibitors compared to lymphoma cell lines. Microtubule inhibitors may be considered potential drug candidates for the treatment of canine HS. On the other hand, it was suggested that alkylating agents, antimetabolites, antitumor antibiotics and topoisomerase inhibitors might not be effective as treatment of HS. Moreover, the expression levels of 16 genes related to drug resistance were examined to elucidate the resistance mechanism toward the antitumor agents used in this study. Finally, the low expression level of TP53 in HS cell lines might play a role in the resistance against a diverse range of chemotherapeutic agents. Although the expression level and mutation of various genes related to drug resistance were examined in cultured canine HS cell lines in this study, similar analyses of selected genes will be needed using primary tumor cell samples obtained from dog patients suffered from HS. The present study provided insights into the mechanisms of chemosensitivity / chemoresistance of canine HS cell lines which can be further investigated to gain further information regarding the pharmacokinetics, safety and efficacy of candidate drugs in treating dogs with HS.

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FIGURE LEGENDS

Fig. 1. Graphs illustrating the IC$_{50}$ values for (a) melphalan, (b) nimustine hydrochloride, (c) methotrexate, (d) cytarabine, (e) vincristine sulfate, (f) vinblastine sulfate, (g) paclitaxel, (h) WSMP, (i) doxorubicin and (j) etoposide. CHS-1, CHS-2, CHS-3 and DH82: canine HS cell lines. CLBL-1 and Ema: canine lymphoma cell lines.

Fig. 2. Graphs illustrating the mRNA expression levels of (a) ABCB1, (b) ABCG2, (c) TP53, (d) p21$^{waf1}$ and (e) MSH6. All data represent the mean ± SD of three independent experiments. *$p$<0.05 (one-way ANOVA followed by Tukey’s post-hoc test).

Fig. 3. Schematic diagram of the locations of the mutations of TP53 gene found in this study. Dark boxes represent the coding exons of the TP53 gene. Open boxes represent the non-coding regions, and open boxes with dotted line represent the deletion of exons. The mutations identified in this study are represented by arrows under the TP53 gene map.
Fig. 4. Efflux of the Rhodamine 123 dye was obvious in the Ema, whereas its efflux was not observed in other cell lines (CHS-1, CHS-2, CHS-3, DH82 and CLBL-1). The solid line, dashed line and dotted line represent the histograms of negative control (N.C), Rhodamine 123 only and Rhodamine 123 with Cyclosporine (Cs), respectively.

Fig. 5. In Western blot analysis, the expression of P-gp was detected in CHS-1, DH82 and Ema, whereas no signal was detected in CHS-2, CHS-3 and CLBL-1.
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Table 1. The IC$_{50}$ values for the 6 cell lines

| Drug examined                  | CHS-1  | CHS-2  | CHS-3  | DH82   | CLBL-1 | Ema     |
|-------------------------------|--------|--------|--------|--------|--------|---------|
| Melphalan                     | 36 µg/ml | 49 µg/ml | 29 µg/ml | 65 µg/ml | 158 n g/ml | 689 n g/ml |
| Nimustine hydrochloride       | 398 µg/ml | 467 µg/ml | 190 µg/ml | 587 µg/ml | 1.42 µg/ml | 876 n g/ml |
| Methotrexate                  | >100 µg/ml | 8.56 µg/ml | 495 n g/ml | >100 µg/ml | 7.10 n g/ml | 17.3 n g/ml |
| Cytarabin                     | 14.4 µg/ml | 40.8 µg/ml | 9.29 µg/ml | 142 µg/ml | 91.7 n g/ml | 736 n g/ml |
| Vincristine sulfate           | 2.53 n g/ml | 2.69 n g/ml | 1.77 n g/ml | 2.66 n g/ml | 1.86 n g/ml | 52.4 n g/ml |
| Vimblastine sulfate           | 2.47 n g/ml | 2.78 n g/ml | 1.75 n g/ml | 2.42 n g/ml | 12.0 n g/ml | 39.7 n g/ml |
| Paclitaxel                    | 53.2 n g/ml | 47.1 n g/ml | 23.8 n g/ml | 58.4 n g/ml | 133 n g/ml | 263 n g/ml |
| WSMP                          | 46 n g/ml | 92.8 n g/ml | 15.6 n g/ml | 19.2 n g/ml | 12.3 n g/ml | 85.5 n g/ml |
| Doxorubicin hydrochloride     | 187 n g/ml | 343 n g/ml | 95.0 n g/ml | 113 n g/ml | 24.8 n g/ml | 69.5 n g/ml |
| Etoposide                     | 3.71 µg/ml | 8.18 µg/ml | 755 n g/ml | 2.19 µg/ml | 67.5 n g/ml | 230 n g/ml |
Fig. 1

(a) Melphalan

(b) Nimustine hydrochloride

(c) Methotrexate

(d) Cytarabine

(e) Vincristine sulfate

(f) Vinblastine sulfate
Fig. 2

(a) *ABCB1*

(b) *ABCG2*

(c) *TP53*

(d) *p21^{waf1}*

(e) *MSH6*
**CHS-1**
- TAT to TAG
- Try^{326} to stop codon

**CHS-2**
- GTG (Val^{206}) deletion

**CLBL-1**
- GCG to GTG
- Ala^{125} to Val

**DH82**: deletion of exon 2 to 11

**CHS-3 and Ema**: no mutation
Fig. 4

**Graphs**

- **CHS-1**
  - **Number of cells**
  - **Fluorescence intensity**

- **CHS-2**
  - **Number of cells**
  - **Fluorescence intensity**

- **CHS-3**
  - **Number of cells**
  - **Fluorescence intensity**

- **DH82**
  - **Number of cells**
  - **Fluorescence intensity**

- **CLBL-1**
  - **Number of cells**
  - **Fluorescence intensity**

- **Ema**
  - **Rh + Cs**
  - **Rh alone**
  - **N.C**
|       | CHS-1 | CHS-2 | CHS-3 | DH82 | Ema | CLBL-1 |
|-------|-------|-------|-------|------|-----|--------|
| **P-gp** | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| **β-actin** | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| Gene     | Forward Sequence  | Reverse Sequence  | Amplicon Size (bp) | GenBank Accession Number |
|----------|-------------------|-------------------|--------------------|--------------------------|
| MSH2     | 5'-CCTTTTCCAGGGTTCTGTG-3' (1018-1037) | 5'-CCTGAGGGGTTTTGCGATTAA-3' (1078-1097) | 80 | XM_538482.4 |
| MSH3     | 5'-CAAGACTCTGCTTCCCCGTCC-3' (904-923) | 5'-CTAGCAGCTGGCTCCTGTG-3' (1009-1028) | 125 | XM_005618164.1 |
| MSH6     | 5'-GGCTTGCTAATCTTCCAGAGG-3' (3889-3909) | 5'-CAGGCAAACTTCCCCGAAATA-3' (3973-3992) | 104 | XM_531814.4 |
| MLH1     | 5'-GAAAGGACATGACAGCA-3' (1246-1265) | 5'-TGTTATGACGACATCTGTCG-3' (1350-1369) | 124 | XM_005634229.1 |
| PMS2     | 5'-GCAAGGCGCTCACTGCTCCTCCC-3' (753-772) | 5'-GAGAGATGAAGCCCGAGATG-3' (849-868) | 99 | NM_001003210.1 |
| ABCB1    | 5'-CCATCTGGAGGAGGAATGA-3' (387-406) | 5'-AGGCACCAGAATGAACCT-3' (466-485) | 122 | NM_001002971.1 |
| ABC1     | 5'-TGATCTGCTGCTTCTGTCC-3' (556-575) | 5'-ACCCGTGTAGACCAAAATG-3' (668-687) | 132 | NM_001002971.1 |
| LRP      | 5'-ATATCTCCGAGCTGTTTG-3' (773-792) | 5'-CGGTCACCTGCTGAGTC-3' (873-892) | 120 | NM_001287146.1 |
| ABCG2    | 5'-AAGATCCGGCATGGATATCG-3' (400-419) | 5'-CCCATCGGACCCTACATGTG-3' (486-505) | 106 | NM_001048021.1 |
| TP53     | 5'-CAGTGTTGTGTTGTGGCTTCTATG-3' (622-641) | 5'-GGAGTCTTCCAGGGTGATG-3' (741-760) | 139 | NM_001003210.1 |
| p21  | 5'-GCGATGGAACTTTGACTTCG-3' | 5'-GCAGGGGACCTTCCCAGCAG-3' (968-987) | 95 | AJ830019 (partial) |
| Bcl-2    | 5'-AGTACCGGAAACCCGAGACATCTC-3' (968-987) | 5'-CCCTCAGAGAGAGCCCGAGGAG-3' (1068-1087) | 120 | NM_001002949.1 |
| MGMT     | 5'-TGACTGAGATAAGGGCTGCAC-3' (140-159) | 5'-TCAGCGAAATAGGCCGATCC-3' (265-284) | 145 | NM_001003376.1 |
| GSTA4    | 5'-CATGGGGAGAGAGGCTTAAA-3' (436-455) | 5'-GATATGCTGGCAAGGCTCAG-3' (524-543) | 108 | NM_001252224.1 |
| GSTP1    | 5'-GGAGACCTCCACCTGTTACCA-3' (259-278) | 5'-GATCCTCCACACCATCTGTC-3' (363-382) | 124 | NM_001252167.1 |
| Survivin | 5'-CCTGGGCAGCTACCTCAAG-3' (90-109) | 5'-AGTGGGCCAGTGGATGAG-3' (189-209) | 119 | NM_001003348.1 |
Supplementary file 2. Detail information on the real-time RT-PCR procedure

Total RNA was extracted from cells using the Illustra RNAspin Mini Kit (GE Healthcare UK Ltd, Little Chalfont, England), while reverse transcription for cDNA synthesis was performed using the ReverTra Ace® qPCR RT Kit (TOYOBO, Tokyo, Japan). Concentrations of the cDNAs were adjusted to 50 µg/ml.

For normalization of cDNA, candidate internal reference genes, including Glyceraldehyde-3-phosphate dehydrogenase (G3PDH), Hydroxymethyl-bilane synthase (HMBS), Ribosomal protein L13a (RPL13A), Ribosomal protein L32 (RPL32), and TATA box binding protein (TBP), were selected and quantified, and their accuracies and suitability as internal controls were analyzed using RefFinder (http://www.leonxie.com/referencegene.php). Primer pairs for the five candidate internal reference genes used in this study have previously been described [35].

Real-time PCR was performed using the Thermal Cycler Dice Real Time System TP800 (Takara Bio, Shiga, Japan) with THUNDERBIRD SYBR® qPCR Mix (TOYOBO) according to the manufacturer’s instructions. The amount of cDNA used in a PCR mixture was 100 ng. The cDNA samples were activated at 95°C for 60 s, then subjected to 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 30 s. After 40 cycles, the samples were run using the dissociation protocol to verify
the presence of a single melting peak. Data were expressed as mean C_T values of experiments performed in triplicate. C_T values were determined using the second derivative maximum method, in which a C_T value was expressed as the cycle number at the location where the second derivative was at its maximum value.

For each drug-resistance gene and two internal reference genes, an assay-specific standard curve was prepared, using serial (10x) dilutions of cDNA from one of the cell lines (CHS-1 for ABCB1 and CLBL-1 for other genes). To prepare the standard curves, the relative quantity of cDNA for each factor and the two internal reference genes, in 100 ng of cDNA, was defined as 10^5. Each standard curve was examined in triplicates. The relative quantities were calculated by plotting C_T values against the standard curves. Normalization of the amount of cDNA samples was performed by calculating the ratio of the relative quantity of cDNA for each factor to those of HMBS and TBP. All samples were examined independently in three separate experiments. The specificities of all PCR amplicons were confirmed by melting curve analysis.
### Supplementary file 3. Sequences of primer pairs used in sequence analysis of *TP53*

| **TP53** | Forward primer | Reverse primer | Exon scanned | Amplicon size (bp) |
|----------|----------------|----------------|--------------|-------------------|
| Fragment A | 5’-ATGCAAGAGCCACACAGTCAG-3’ | 5’-GACCTCCCCACACCCAGT-3’ | Exon 2-3 | 277 |
| Fragment B | 5’-CTTGACTCTGGTCTC GCC-3’ | 5’-GCCAGCCCCATGGA AACC-3’ | Exon 4 | 304 |
| Fragment C | 5’-GACCTGTCCATCTGTCCT-3’ | 5’-AGACCCCTCAGATGCC AA-3’ | Exon 5-6 | 434 |
| Fragment D | 5’-ACCCCTGGCCCTACCTT CT-3’ | 5’-CTCCCTTCACCTCCTC TTGT-3’ | Exon 7-8 | 565 |
| Fragment E | 5’-GCTCAAAACATA CTCTTCTCT-3’ | 5’-TGCCCTTATCTGTTCCT CCCC-3’ | Exon 9 | 150 |
| Fragment F | 5’-AATGGTACTGTGG GTCCTC-3’ | 5’-CAAGCCGGCCAGGCTCA-3’ | Exon 10 | 92 |
| Fragment G | 5’-CTCCCACTTTGCTA ATATCGT-3’ | 5’-TGAGGGGTGTGCGT GTTGG -3’ | Exon 11 | 167 |

| **MMP3** | Forward primer | Reverse primer | Amplicon size (bp) |
|----------|----------------|----------------|-------------------|
| | 5’-AGTGGGAGTCTGCTTGAGA-3’ | 5’-TGTTGCTTTGGGT ACTTTC-3’ | 184 |
| | (7486-7505)$^b$ | (7650-7669)$^b$ | |

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$b$ The numbers in parentheses indicate the nucleotide numbers registered in GenBank (NC_006587).
Supplementary file 4. Sequences of primer pairs used in sequence analysis of *ABCB1*

| *ABCB1* | Forward primer | Reverse primer | Amplicon size (bp) |
|---------|----------------|----------------|--------------------|
| Fragment 1 | 5'-CTTCCCAAATCCCTTCTCG-3' | 5'-CCCCCACTATAAAACC GGTA-3' | 676 |
| Fragment 2 | 5'-TTTCATGCTATCATGCGACAG-3' | 5'-GCTGTCAATGGCTTGGTTTATTTG-3' | 678 |
| Fragment 3 | 5'-ACCTCCCTGTCCTCCACGAG-3' | 5'-CTTGGGGTTGCGAACCAG-3' | 699 |
| Fragment 4 | 5'-GCCACCAGATGCTGAA-3' | 5'-CAGCTTCAGAATCCCTCCAGAA-3' | 669 |
| Fragment 5 | 5'-TGTCACAATGCAGACAAAGG-3' | 5'-GCCTGTCCCAAGATTTGCTA-3' | 664 |
| Fragment 6 | 5'-CACTGGCCGCTTCCGATA-3' | 5'-GGCTTTGGCAGTATAGTCAGGAG-3' | 652 |
| Fragment 7 | 5'-GAAACTCTTTGAGGAAAGCACA-3' | 5'-GGAGTGTCTCGATGAAGTG-3' | 699 |
| Fragment 8 | 5'-GGCAAAGAGATAAACGCCT-3' | 5'-TGATCTTCAGTTGCAGCAAGA-3' | 692 |
Supplementary file 5. Relative quantities\textsuperscript{a)} of mRNA of 16 drug resistance genes in the

| Drug resistance gene | CHS-1 | CHS-2 | CHS-3 | DH82 | CLBL-1 | Ema  |
|----------------------|-------|-------|-------|------|--------|------|
| ABCB1                | 9900  | 390   | 5.9   | 4200 | 1      | 49000|
| ABCC1                | 1.12  | 0.84  | 2.97  | 2.46 | 1      | 1.66 |
| ABCG2                | 8.62  | 7.96  | 2.5   | 11   | 1      | 0.11 |
| LRP                  | 0.55  | 0.64  | 0.86  | 0.71 | 1      | 0.01 |
| p53                  | 0.04  | 0.005 | 0.68  | -    | 1      | 0.97 |
| p21\textsuperscript{waf1} | 508  | 838   | 2300  | 1370 | 1      | 44.1 |
| Bcl-2                | 0.11  | 1.65  | 0.49  | 0.08 | 1      | 1.09 |
| Survivin             | 1.07  | 2.5   | 1.51  | 2.71 | 1      | 0.99 |
| GSTA4                | 1.6   | 1.36  | 2.27  | 2.62 | 1      | 5.26 |
| GSTP1                | 3.05  | 1.5   | 0.02  | 6.38 | 1      | 1.27 |
| MGMT                 | 3.35  | 1.23  | 3.34  | 0.64 | 1      | 3.45 |
| MSH2                 | 0.74  | 0.51  | 0.53  | 1.25 | 1      | 2.23 |
| MSH3                 | 0.51  | 0.83  | 1.55  | 0.79 | 1      | 0.87 |
| MSH6                 | 0.44  | 0.24  | 0.32  | 0.37 | 1      | 1.51 |
| MLH1                 | 0.76  | 0.96  | 0.97  | 0.68 | 1      | 0.34 |
| PMS2                 | 0.92  | 0.67  | 1.4   | 1.56 | 1      | 0.91 |

\textsuperscript{a)} All expression levels were normalized to those of the same mRNA in CLBL-1.