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Establishment and Characteristics of a Novel Mantle Cell Lymphoma-derived Cell Line and a Bendamustine-resistant Subline

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Abstract. *Background/Aim: Bendamustine hydrochloride (BH) is a key therapeutic agent for mantle cell lymphoma (MCL), while the mechanism underlying BH-resistance has not been verified. Materials and Methods: We compared molecular/biological characteristics of a newly-generated MCL-derived cell line KPUM-YY1 and its BH-resistant subline KPUM-YY1R. Results: The growth-inhibitory IC_{50} for BH was 20 μM in KPUM-YY1 cells, while cell proliferation was not inhibited by up to 60 μM BH in KPUM-YY1R cells. Compared to KPUM-YY1 cells, gene expression profiling in KPUM-YY1R cells revealed up-regulation of 312 genes, including ABCB1 encoding P-glycoprotein (P-gp), and microsomal glutathione S-transferase 1 (MGST1). Addition of either a P-gp inhibitor or a GST inhibitor, at least partly, restored sensitivity to BH in KPUM-YY1R cells. In addition, KPUM-YY1R cells showed cross-resistance against various anti-MCL chemotherapeutics. Conclusion: BH resistance is mediated by overlapping mechanisms with overexpression of ABCB1 and MGST1, and is potentially accompanied by multidrug resistance in MCL.*

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Key Words: Mantle cell lymphoma, bendamustine, resistance, ABCB1, MGST1.

Mantle cell lymphoma (MCL) is one of the most difficult-to-treat subtypes of non-Hodgkin's lymphoma (NHL), despite recent advances in therapeutic strategies, including immunochemotherapy incorporating rituximab (RIT) and genotoxic agents, ibrutinib, bortezomib (BTZ), or high-dose chemotherapy followed by autologous stem cell transplantation (1-4). Amongst various cytotoxic agents, bendamustine hydrochloride (BH) is one of the most promising central drugs in the treatment for MCL. BH exerts an anti-lymphoma effect through complex and overlapping mechanisms, including induction of apoptosis *via* a DNA-damage stress response, inhibition of mitotic checkpoints, and induction of mitotic catastrophe (5, 6).

In a clinical setting, BH plus rituximab increases progression-free survival (PFS) and has fewer toxic effects than the standard R-CHOP regimen (RIT, cyclophosphamide (CPA), doxorubicin (DOX), and vincristine (VCR)) in previously untreated patients with MCL (5). BH also has clinical activity in patients with relapsed NHL, including those refractory to other alkylating agents and purine analogs (5, 6). However, relapse occurs in most treated cases of MCL (7), and little is known about the mechanisms of resistance to BH. In this study, we investigated the molecular profiles of a novel MCL cell line with acquired BH resistance, with the goal of identifying the mechanism of resistance as a basis for development of a strategy to overcome BH resistance in treatment of MCL.

Materials and Methods

Establishment of cell lines. Primary lymphoma cells were isolated from peripheral blood of a MCL patient with informed consent, and were cultured in RPMI-1640 supplemented with 10% heat-

inactivated fetal bovine serum, 2 mmol/l L-glutamine, and penicillin/streptomycin in a highly humidified atmosphere of 5% CO_2 and 95% air at 37˚C. A novel cell line designated as KPUM-YY1 was established after continuous culture for several months. KPUM-YY1 cells were subjected to continuous exposure to BH with gradual escalation of the concentration up to 50 μM, and a BH-resistant subline KPUM-YY1R was established. Subsequently, KPUM-YY1R cells were cultured and maintained by passage in a culture medium containing 50 μM BH for over years. A human MCL-derived cell line, Jeko-1, was also used in some of the analyses below. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and with the approval of the institutional ethical review board of Kyoto Prefectural University of Medicine.

Cytogenetic and genome copy number analyses. Double color-fluorescence in situ hybridization (DC-FISH) and spectral karyotyping were performed as described previously (8, 9). The karyotype was described in accordance with the International System for Human Cytogenetic Nomenclature ISCN 2013 (10). Vysis IGH/CCND1 DF FISH and IGH/C-MYC DF FISH probe kits (Abbott Japan, Tokyo, Japan) were utilized for DC-FISH. A DNA gain and loss assay based on a SNP array (GeneChip Human Mapping 6.0 array; Affymetrix, Santa Clara, CA, USA) was performed on genomic DNA purified from KPUM-YY1 and KPUM-YY1R cells. The CNAG3.5.1 program was used for analysis of SNP array data to determine total copy numbers (11, 12).

Microarray analysis and signal pathway analysis. Comprehensive gene expression analysis was performed to compare gene expression profiles in KPUM-YY1 and KPUM-YY1R cells, using GeneChip arrays, a GeneChip Scanner 3000, and array data analysis with GeneChip software, v.1.0 (Affymetrix). Genes with at least a 1.5-fold difference in expression between KPUM-YY1 and KPUM-YY1R cells were considered positive. For signal pathway analysis, data were analyzed with Ingenuity software (Ingenuity Systems, Mountain View, CA, USA).

Assay for TP53 mutational status. *TP53* gene mutational status was analyzed as described elsewhere (13).

Assays for growth inhibition and drug combination. Cells were treated with various concentrations of BH for 96 h, and cytarabine (CA), DOX, etoposide (ETP), fludarabine (FLU), VCR (Sigma-Aldrich, St. Louis, MO), ibrutinib (Selleck Chemicals, Houston, TX), mafosfamide (the active metabolite of CPA, Santa Cruz Biotechnology, Dallas, TX, USA), lenalidomide (LEN) (Celgene Corp., Summit, NJ), melphalan, or BTZ (provided by the Screening Committee of Anticancer Drugs) for 48 h, and cell viability was evaluated using a modified MTT assay in Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Cyclosporine A (Wako Pure Chemical Industries, Ltd., Osaka, Japan), verapamil (Sigma-Aldrich), PSC833, and C-4 (Santa Cruz Biotechnology) were utilized as inhibitors for ABCB1, and ethacrynic acid was used as a glutathione-S-transferase (GST) inhibitor (Sigma-Aldrich). Data are shown as the mean±standard error of three independent experiments.

Reverse-transcription-polymerase chain reaction (RT-PCR). RT-PCR analysis was performed as described previously (12), using the following primers: ABCB1-S, 5'-CAC GTG GTT GGA AGC TAA CC-3' and ABCB1-A, 5'-GAA GGC CAG AGC ATA AGA TGC-3' for detection of *ABCB1* expression; MGST1-S, 5'-ATT GGC CTC CTG TAT TCC TTG and MGST1-A, TAA TTC CTC TGC TCC CCT CC for detection of *MGST1* expression, and β-actin-S, 5'-CTT CTA CAA TGA GCT GCG TG-3' and β-actin-A, 5'-TCATGAGGTAGTCAGTCAGG-3' for detection of β-actin expression as an internal control.

Western blotting. Western blotting was performed as described previously (14), using a mouse monoclonal primary antibody for ABCB1 (D-11 clone; Santa Cruz Biotechnology) and an antibody to β-actin (Sigma-Aldrich) as an internal control.

Results

Establishment and cytogenetic features of KPUM-YY1 cells. A 60-year-old male patient was diagnosed with MCL clinical stage IVA in the Ann Arbor staging system. The patient was treated with chemotherapies including R-CHOP, Hyper-CVAD/MTX-CA (CPA, VCR, DOX, dexamethasone (DEX), high-dose methotrexate (MTX) and CA), CHASE (CPA, high-dose CA, DEX, ETP), cladribine, BTZ, irinotecan, L-asparaginase, gemcitabine, and BH for repeated relapse and remission, and eventually died of MCL progression after 17 years of treatment. A novel cell line, KPUM-YY1, was developed from circulating primary lymphoma cells collected from the patient in the terminal phase. KPUM-YY1 cells were morphologically round and medium in size with various forms of deformed nuclei (Figure 1A). These cells were maintained as a suspension culture in conventional culture medium. DC-FISH for KPUM-YY1 cells identified the presence of fusion genes of *immunoglobulin heavy chain (IGH)* and *cyclin D1* (Figure 1B) and of *IGH* and *c-MYC* (Figure 1C). SKY analyses further revealed that KPUM-YY1 cells harbored complex karyotypic abnormalities, including a three-way translocation t(8;14;11)(q24;q32;q13), but not del17p (Figure 1D). This three-way translocation was also observed in patient-derived primary lymphoma cells (Data not shown).

Development of a BH-resistant subline KPUM-YY1R and its cytogenetic and molecular characteristics. The patient exhibited clinical resistance to BH, but growth of KPUM-YY1 cells was inhibited by BH, with an IC_{50} of 20 μM in 96-hour treatment *in vitro* (Figure 2A). To investigate the mechanisms underlying resistance to BH, we sought to establish a BH-resistant subline of KPUM-YY1. Continuous exposure to BH with gradual escalation of the BH concentration from 5 to 50 μM for 8 months generated a BH-resistant subline, KPUM-YY1R, that was not inhibited by up to 60 μM BH and proliferated at 50.0 μM BH (Figure 2A and B).

To dissect the cytogenetic and molecular characteristics of this subline, including those associated with BH resistance, comparative analyses of cytogenetics and gene expression profiles (GEPs) in KPUM-YY1 and KPUM-YY1R cells were performed using SKY, genome copy number, and

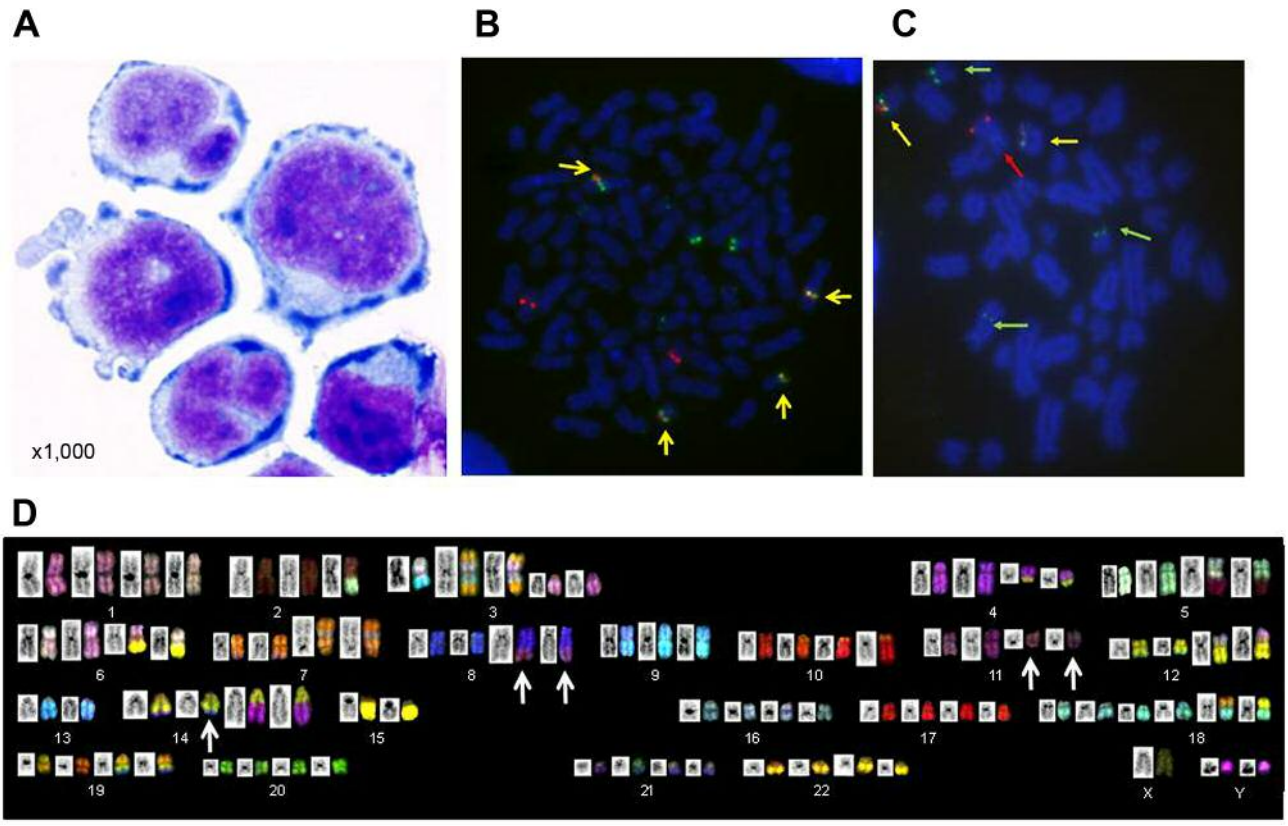

Figure 1. *Morphological and cytogenetic features of KPUM-YY1 cells. A: Morphology of KPUM-YY1 cells. Cells were cytospinned and stained using Diff-Quik (Sysmex, Kobe, Japan), and the morphology was examined under a light microscope (magnification ×1,000). B: Metaphase double color (DC)-fluorescence in situ hybridization (FISH) analysis for immunoglobulin heavy chain (IgH) and cyclin D1 (CCND1) in KPUM-YY1 cells. Red and green signals indicate CCND1 at 11q13 and IgH at 14q32, respectively. Yellow signals (arrows) indicate fusion of red and green signals, confirming the reciprocal translocation IgH/CCND1. C: Metaphase DC-FISH analysis for IgH and c-MYC in KPUM-YY1 cells. Red and green signals indicate c-MYC at 8q24 (red arrows) and IgH at 14q32 (green arrows), respectively. Yellow signals (arrows) indicate fusion of red and green signals, confirming the reciprocal translocation IgH/c-MYC. D: SKY analysis of KPUM-YY1 cells. The representative karyotype of KPUM-YY1 is 4n: 88, YY, inv(X)(p11q28), t(2;5)(q21;q15), -2,del(3)(p12)x2,+der(3)t(3;13)(q12;q14), der(3)(7qter→7q22::3p21 →3q21::13::7q11.2→7qter)x2, t(4;14)(q12;q32.1)x2, der(5)t(2;5), der(6)t(6;8)(p25;q24)x2, der(6)t(6;15)(q13;q11)x2, add(7)(q22)x2, der(7)(7qter→7q11.2::8::7p22→7q11.2::7p22→7pter)x2, t(8;14;11)(q24;q32;q13)x2, dup(9)(p13p24)x3, -9, der(10)t(10;19) (p15;q13) x2, t(12;18)(q15;p11.2)x2, der(12)t(3;12)(p21;p11.2)x2, -13x2, -15x2, +18x2, der(19)(19pter→19q13.1::14q24→14q32:: 8q24→8qter)x2. Arrows indicate a three-way translocation t(8;14;11)(q24;q32;q13).*

expression microarray analyses. Several cytogenetic abnormalities were identified in KPUM-YY1R cells that were not present in parental KPUM-YY1 cells in SKY and genome array analyses (Table I, Figure 2C and D).

In GEP analyses, 312 genes were up-regulated more than 1.5-fold and 160 genes were down-regulated to less than 0.67-fold in KPUM-YY1R cells compared with KPUM-YY1 cells (Data not shown). Genes upregulated in KPUM-YY1R cells included *ABCB1* (1.79-fold), and the Ingenuity canonical signal pathway analysis based on the GEP results suggested that KPUM-YY1R cells harbored distinct gene expression patterns in the glutathione S-transferase (GST) family, such as *MGST1* (3.23-fold), *GSTA4*, or *GSTM2* (Table II). Indeed, overexpression of *MDR1* and *MGST1* was confirmed by RT-PCR (Figure 2E) and an increase of P-gp was found at the protein level (Figure 2F). Eleven of 472 genes with differential expression in GEP were associated with additional genomic regions identified by SKY and genome copy number analyses in KPUM-YY1R cells (Table I), but none had previously been associated with drug resistance. In addition, TP53 mutation was absent in KPUM-YY1 and KPUM-YY1R cells (data not shown).

ABCB1 and MGST1 are involved in resistance to BH in KPUM-YY1R cells. Based on the above results, we focused on the ABCB1 and GST families as candidate causes of acquisition of resistance to BH in KPUM-YY1R cells. Addition of an inhibitor of ABCB1 protein (5 μM

cyclosporine A, 10 μM verapamil, 5 μM PSC833, or 5 μM C-4) partly restored the sensitivity to BH in KPUM-YY1R cells (Figure 3A, B, C and D). Perhaps consistent with faint expression of ABCB1 protein in parental KPUM-YY1 cells (Figure 2F), addition of ABCB1 inhibitors also enhanced the growth inhibitory effect of BH in KPUM-YY1 cells, although the degree of enhancement was much smaller compared with that in KPUM-YY1R cells. Similarly, addition of 30 μM ethacrynic acid, a GST inhibitor, partly restored sensitivity to BH in KPUM-YY1R cells (Figure 3E). These results indicate the functional significance of upregulation of *ABCB1* and *MGST1* in development of BH resistance in MCL.

Cross-resistance to other chemotherapeutic agents in KPUM-YY1R cells. Compared to parental KPUM-YY1 cells, KPUM-YY1R cells showed partial cross-resistance to DOX, mafosfamide, melphalan, VCR and ibrutinib (Figure 4), whereas sensitivity to cytarabine, ETP, FLU, BTZ, and LEN, which are used in treatment of MCL, were mostly equivalent in the two cell lines (Figure 5).

Discussion

BH is a unique anticancer agent since it structurally consists of three active moieties: an alkylating group, in common with the nitrogen mustard family; a benzimidazole ring, which may act as a purine analog; and a butyric acid sidechain. Therefore, BH may have antitumor effects against various types of cancers. At present, BH is used as a central therapeutic component in both first-line and salvage strategies for various types of low grade B-cell NHLs and for MCL, a usually aggressive and difficult-to-treat disease subtype, in the clinical setting (15-19). Regarding its mechanisms of action, BH alkylates and crosslinks DNA, leading to double-strand breaks (DSBs) followed by p53-dependent and -independent apoptosis in cancer cells, with formation of DSBs that is more powerful and long-lasting than other alkylating agents. In addition, unlike other alkylators, BH can also induce mitotic catastrophe *via* inhibition of mitotic checkpoint regulators such as *polo-like kinase 1, aurora kinase A*, or *cyclin B1* (6, 17, 20, 21). Due to these overlapping mechanisms, BH has only partial *in vitro* cross-resistance with other alkylators, such as CPA or melphalan (17), and has full antitumor activity in anthracycline-resistant and platinum-drug resistant cell lines (20). These findings may explain the efficacy of BH for both treatment-naïve and relapsed patients with indolent NHL, including those who have lost responsiveness to other alkylating agents or purine analogs (5, 20). In addition, BH has been shown to exert synergistic cytotoxicity with BTK inhibitors on MCL-derived cell lines, implicating its further therapeutic application (22).

The molecular mechanism for resistance to BH has not been investigated as intensively as the mechanism of action. However, acquisition of clinical resistance to BH is a critical event that potentially determines the life expectancy of patients with B-cell NHL, and especially MCL, and there is an urgent need to understand the mechanisms underlying resistance to BH. To answer this question, in this study we developed a novel MCL-derived cell line, KPUM-YY1, and a BH-resistant subline, KPUM-YY1R, by continuous *in vitro* exposure to BH, and performed comparative analysis of the molecular profiles of these cells. Using this approach, we found that upregulation of *ABCB1* and *GST*, which occurs commonly in drug resistance mechanisms (23), contributed to acquisition of resistance to BH, and we also showed that acquisition of resistance to BH has a potential risk for acquisition of cross-resistance to anti-lymphoma chemotherapeutics such as DOX, mafosfamide, melphalan, and VCR.

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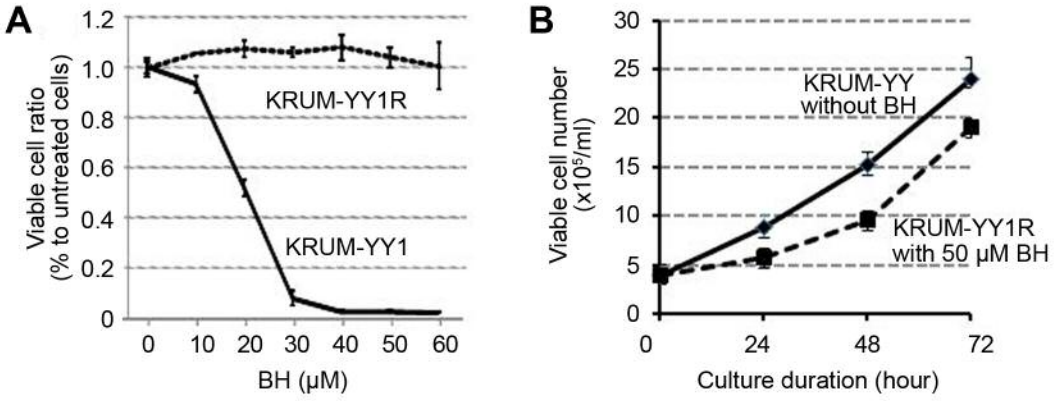
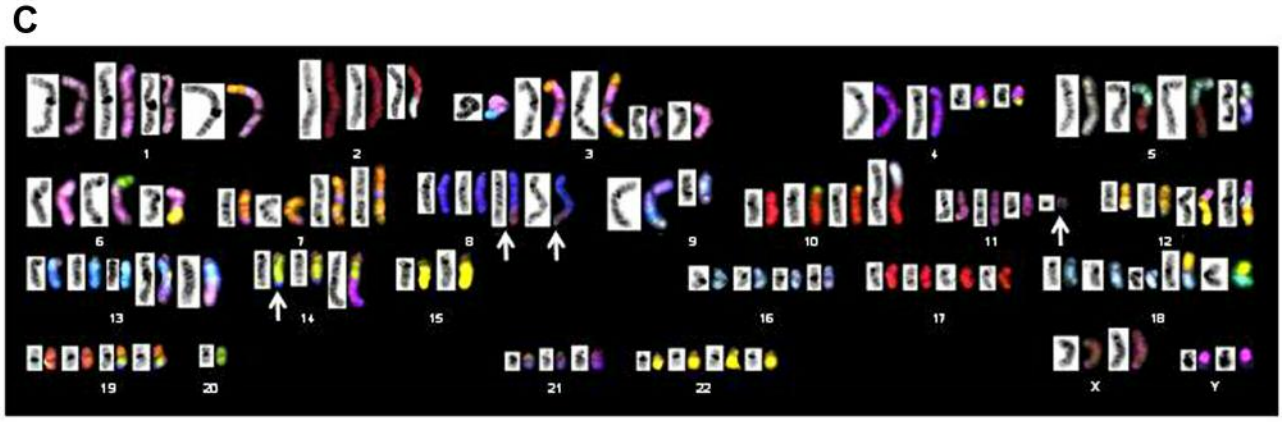
Figure 2. *Cellular and cytogenetic/molecular characteristics of bendamustine (BH)-resistant KPUM-YY1R cells. A: Growth inhibitory effect of BH on KPUM-YY1 and KPUM-YY1R cells. Cells were treated with BH for 96 h and cell viability was evaluated by a modified MTT assay. Data are shown as means+/-standard errors from triplicate experiments. Growth of KPUM-YY1 cells was inhibited by BH in a dose-dependent manner, while growth of KPUM-YY1R cells was not inhibited by 60 μM BH. B: Growth curves of KPUM-YY1 and KPUM-YY1R cells. KPUM-YY1 cells were cultured in normal medium, while KPUM-YY1R cells were grown in culture medium containing 50 μM BH. Cells were stained by Trypan Blue and viable cell numbers were counted under an inverted microscope. C: SKY analysis of KPUM-YY1R cells. The representative karyotype of KPUM-YY1R cells is 4n: 84, YY, inv(X)(p11q28)x2, der(1)t(1;7)(p32;q11.2), t(2;5)(q21;q15), -2, del(3)(p12)x2, +der(3)t(3;13)(q12;q14), der(3)(7qter→7q22::3p21→3q21::13::7q11.2→7qter)x2, t(4;14)(q12;q32.1), der(4)t(4;14), der(5)t(2;5), der(5)t(5;9)(q11;p13), der(6)t(6;15)(q13;q11), der(6)t(6;8)(p25;q24)x2, dic(6;20)(p21;q13), -6, add(7)(q22)x2, der(7)(7qter→7q11.2::8::7p22→7q11.2::7p22→7pter)x2, t(8;14;11)(q24;q32;q13)x2, del(9)(q21), der(9)(9pter→9q22::13::5), -9, -9, der(10)t(10;19)(p15;q13)x2, der(10)(5qter→5q15::2q21→2q13::10p11→10qter), t(12;18)(q15;p11.2)x2, der(12)t(3;12)(p21;p11.2)x2, der(13)t(1;13)(p22;q32)x2, +13, -14, -15, -15, +18, der(19)(19pter→19q13.1::14q24→14q32:: 8q24→8qter)x2, -20, -20, -20, -21. Arrows indicate a three-way translocation t(8;14;11)(q24;q32;q13). D: Genome copy number analysis in KPUM-YY1R cells. A DNA gain and loss assay based on a SNP array was performed on genomic DNA purified from KPUM-YY1R cells. The CNAG3.5.1 program was used for analysis of SNP array data to determine total copy numbers. E: RT-PCR analyses for ABCB1 and MGST1. Transcriptional expression of ABCB1 and MGST1 was examined in KPUM-YY1 cells, KPUM-YY1R cells, normal B lymphocytes, and Jeko-1 cells (MCL-derived cell line). β-Actin was used as an internal control. F: Western blot for ABCB1 protein. ABCB1 protein expression was examined in KPUM-YY1 cells, KPUM-YY1R cells, normal B lymphocytes, and Jeko-1 cells, with β-Actin used as an internal control.*


A
Viable cell ratio (% to untreated cells)
KRUM-YY1R
KRUM-YY1
BH (μM)
B
Viable cell number (x10⁵/ml)
KRUM-YY without BH
KRUM-YY1R with 50 μM BH
Culture duration (hour)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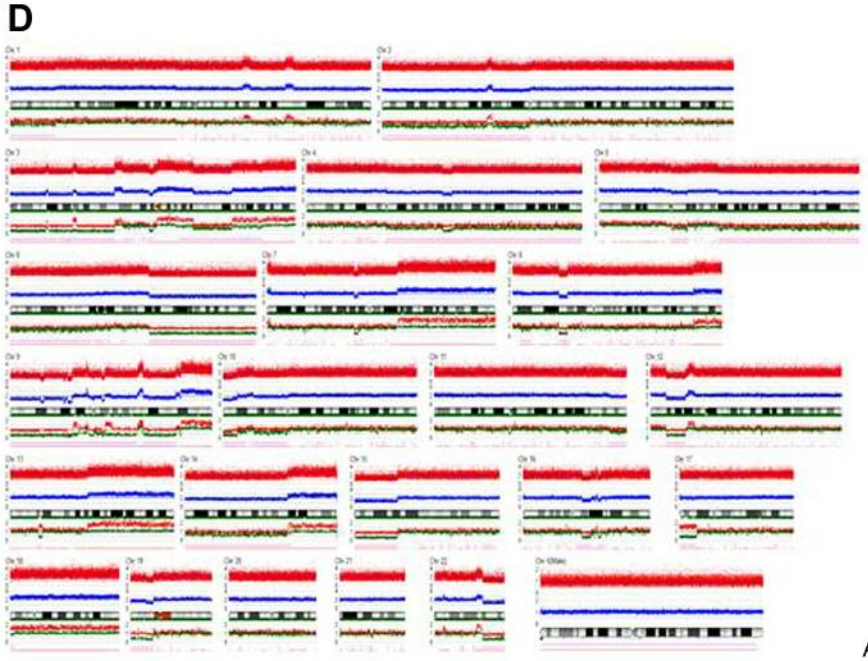
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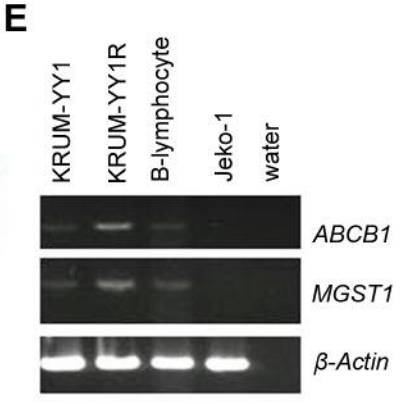
E
KRUM-YY1
KRUM-YY1R
B-lymphocyte
Jeko-1
water
ABCB1
MGST1
β-Actin

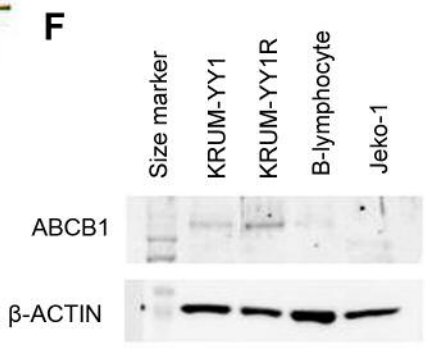
F
Size marker
KRUM-YY1
KRUM-YY1R
B-lymphocyte
Jeko-1
ABCB1
β-ACTIN

Table I. *Comparison of cytogenetic and gene expression analysis between KPUM-YY1 and KPUM-YY1R cells.*

Method	KPUM-YY1	KPUM-YY1R	Differentially expressed genes in GEP	
			Up-regulated	Down-regulated
SKY		der(1)t(1;7)(p32;q11.2)	ZYG11A, CDKN2C, JUN	
		der(5)t(5;9)(q11;p13)	ANKRD18A	CNTNAP3
		der(9)(9pter→9q22::13::5)		
		der(10)(5qter→5q15::2q21→2q13::10p11→10qter)		
		der(13)t(1;13)(p22;q32)		
		-9		
Genome Array		1p13.3-1p35.1 gain	ZYG11A, IFI44, IFI44L SORT1, JUN, MANEAL	
		2q11.2-2q21.2 gain		
		3q13.11 gain		
		4q12-4q22.1 loss	IGJ	
		5q11.1-5q12.1 loss		
		10p11.110p13 loss	LYZL1	
		10p14 loss		
		10p15.1-10p15.3 loss		
	11p14.3 loss			
		16q12.2-16q21 loss		
		16q21 loss		
		16q21 mgain		

Table II. *Top ingenuity canonical pathways enriched by genes that were significantly differentially up-regulated in KPUM-YY1R cells.*

Ingenuity canonical pathways	-log(*p*-Value)	Ratio	Molecules
Epithelial adherence junction signaling	2.60E+00	5.19E -02	MAGI1, LMO7, LEF1, TCF3, NOTCH1, ACVR2A, TCF7L2, CTNND1
Factors promoting cardiogenesis in vertebrates	2.53E+00	6.06E -02	FZD6, LEF1, MEF2C, TCF3, ACVR2A, TCF7L2
Glutathione - mediated detoxification	2.22E+00	6.82E -02	MGST1, GSTM2, GSTA4
Autoimmune thyroid disease signaling	1.79E+00	4.84E -02	CD80, TSHR, CD86
Mouse embryonic stem cell pluripotency	1.71E+00	5.05E -02	ID1, FZD6, LEF1, TCF3, TCF7L2
Human embryonic stem cell pluripotency	1.70E+00	3.70E -02	S1PR4, PDGFA, FZD6, LEF1, TCF3, TCF7L2
Aryl hydrocarbon receptor signaling	1.66E+00	3.51E -02	MGST1, GSTM2, JUN, NFIA, GSTA4, NRIP1
Role of Wnt/GSK-3 signaling in the pathogenesis of influenza	1.65E+00	4.82E -02	FZD6, LEF1, TCF3, TCF7L2
Type I diabetes mellitus signaling	1.64E+00	4.13E -02	CD3G, CD80, SOCS6, SOCS2, CD86
Systemic lupus erythematosus signaling	1.59E+00	2.73E -02	CD3G, RNPC3, JUN, CD80, RNU4 CD86, RNU5A -1
Basal cell carcinoma signaling	1.58E+00	5.13E -02	FZD6, LEF1, TCF3, TCF7L2
Thyroid cancer signaling	1.57E+00	6.82E -02	LEF1, TCF3, TCF7L2
Glutathione redox reactions I	1.51E+00	8.33E -02	MGST1, GPX7
CD28 signaling in T helper cells	1.51E+00	3.68E -02	CD3G, PAK1, JUN, CD80, CD86
Nur77 signaling in T lymphocytes	1.41E+00	4.69E -02	CD3G, CD80, CD86
TNFR1 signaling	1.41E+00	5.56E -02	PAK1, JUN, TNFAIP3
CTLA4 signaling in cytotoxic T lymphocytes	1.35E+00	4.17E -02	CD3G, CD80, CD86, PTPN22
Diphthamide biosynthesis	1.32E+00	7.69E -02	DPH5

ABCB1 encodes P-gp, a plasma membrane drug efflux pump that causes resistance to its substrate anticancer agents, such as DOX, VCR, and ETP. Drugs that are potential inducers of P-gp are generally also substrates of this transporter, but recent studies have shown that several agents that are not transportable by P-gp can also induce P-gp overexpression. Moreover, P-gp has effects that are independent of its drug efflux activity, including effects on the function of TP53,

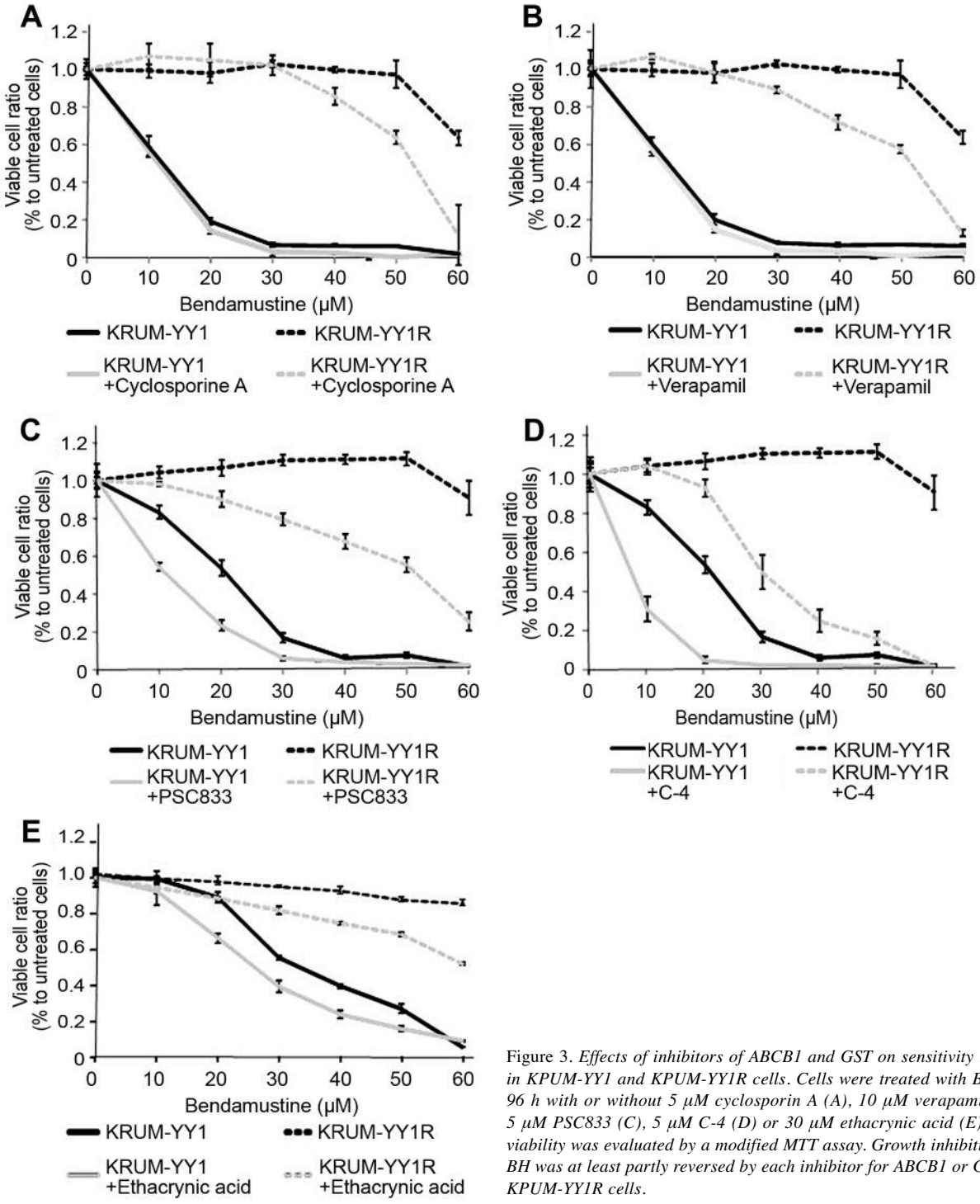

Figure 3. *Effects of inhibitors of ABCB1 and GST on sensitivity to BH in KPUM-YY1 and KPUM-YY1R cells. Cells were treated with BH for 96 h with or without 5 μM cyclosporin A (A), 10 μM verapamil (B), 5 μM PSC833 (C), 5 μM C-4 (D) or 30 μM ethacrynic acid (E). Cell viability was evaluated by a modified MTT assay. Growth inhibition by BH was at least partly reversed by each inhibitor for ABCB1 or GST in KPUM-YY1R cells.*

survivin, caspases, protein expression, glycosylation and phosphorylation, which may collectively induce changes in cell sensitivity to agents that are not P-gp substrates (24-27). Indeed, BH has not been proven to be a substrate for the P-gp drug efflux pump; therefore, we speculate the possible involvement of drug efflux pump-independent mechanisms for the acquisition of resistance to BH in KPUM-YY1R cells. Irradiation-induced P-gp overexpression has been associated

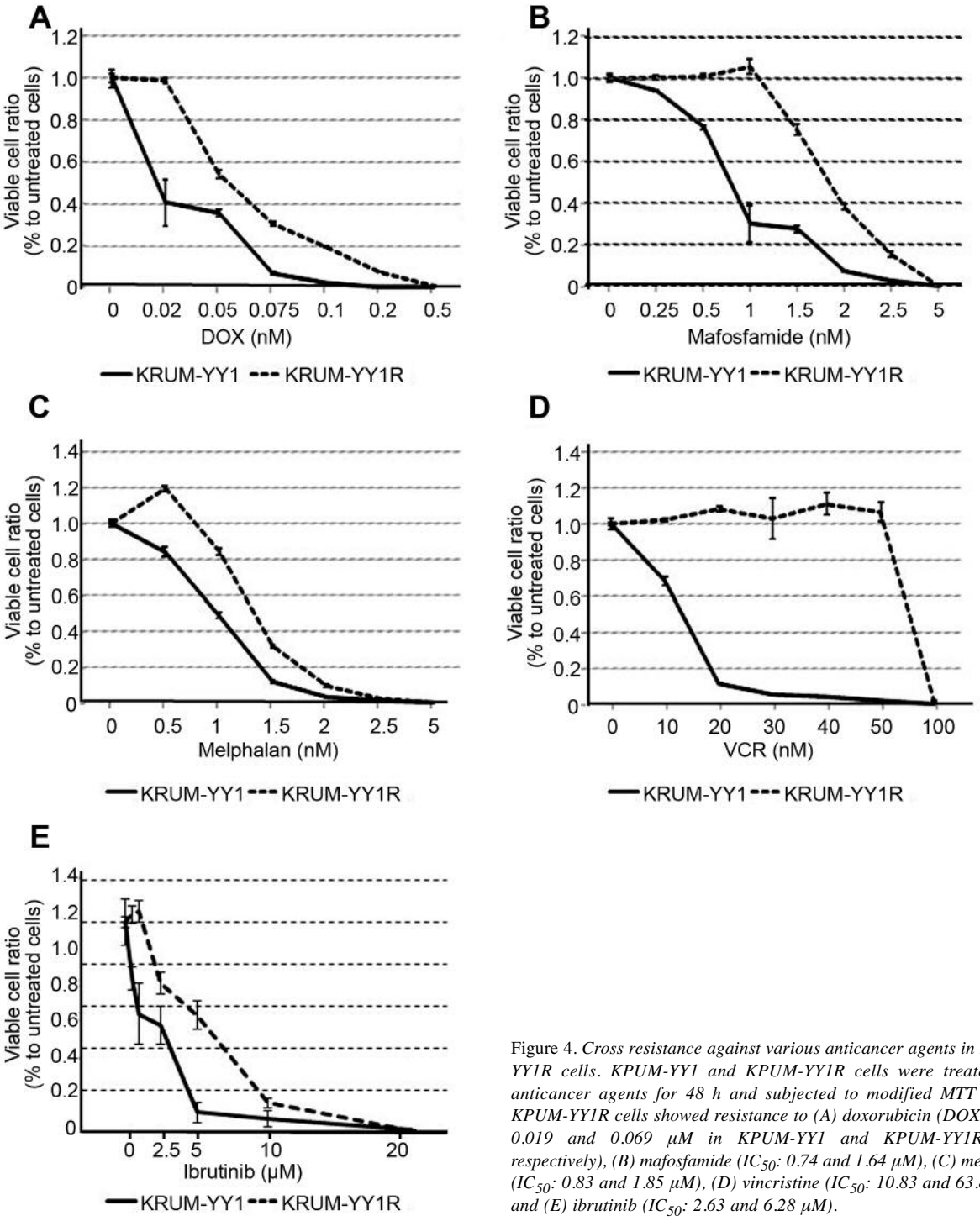

Figure 4. *Cross resistance against various anticancer agents in KPUM-YY1R cells. KPUM-YY1 and KPUM-YY1R cells were treated with anticancer agents for 48 h and subjected to modified MTT assays. KPUM-YY1R cells showed resistance to (A) doxorubicin (DOX) (IC_{50}: 0.019 and 0.069 μM in KPUM-YY1 and KPUM-YY1R cells, respectively), (B) mafosfamide (IC_{50}: 0.74 and 1.64 μM), (C) melphalan (IC_{50}: 0.83 and 1.85 μM), (D) vincristine (IC_{50}: 10.83 and 63.88 nM), and (E) ibrutinib (IC_{50}: 2.63 and 6.28 μM).*

with resistance to BH in colorectal cancer cell lines (28). The pathway through which continuous exposure to BH causes up-regulation of *ABCB1* is uncertain, but resistance to BH is at least partly reversed by addition of P-gp antagonists, as for resistance to anthracyclines or vinca alkaloids (29, 30).

Conjugation with glutathione mediated by GSTs is a major route of bendamustine metabolism (31). GSTs are a complex multigene family of enzymes that detoxify electrophilic xenobiotics such as alkylating agents, such that overexpression of GSTs is associated with resistance to

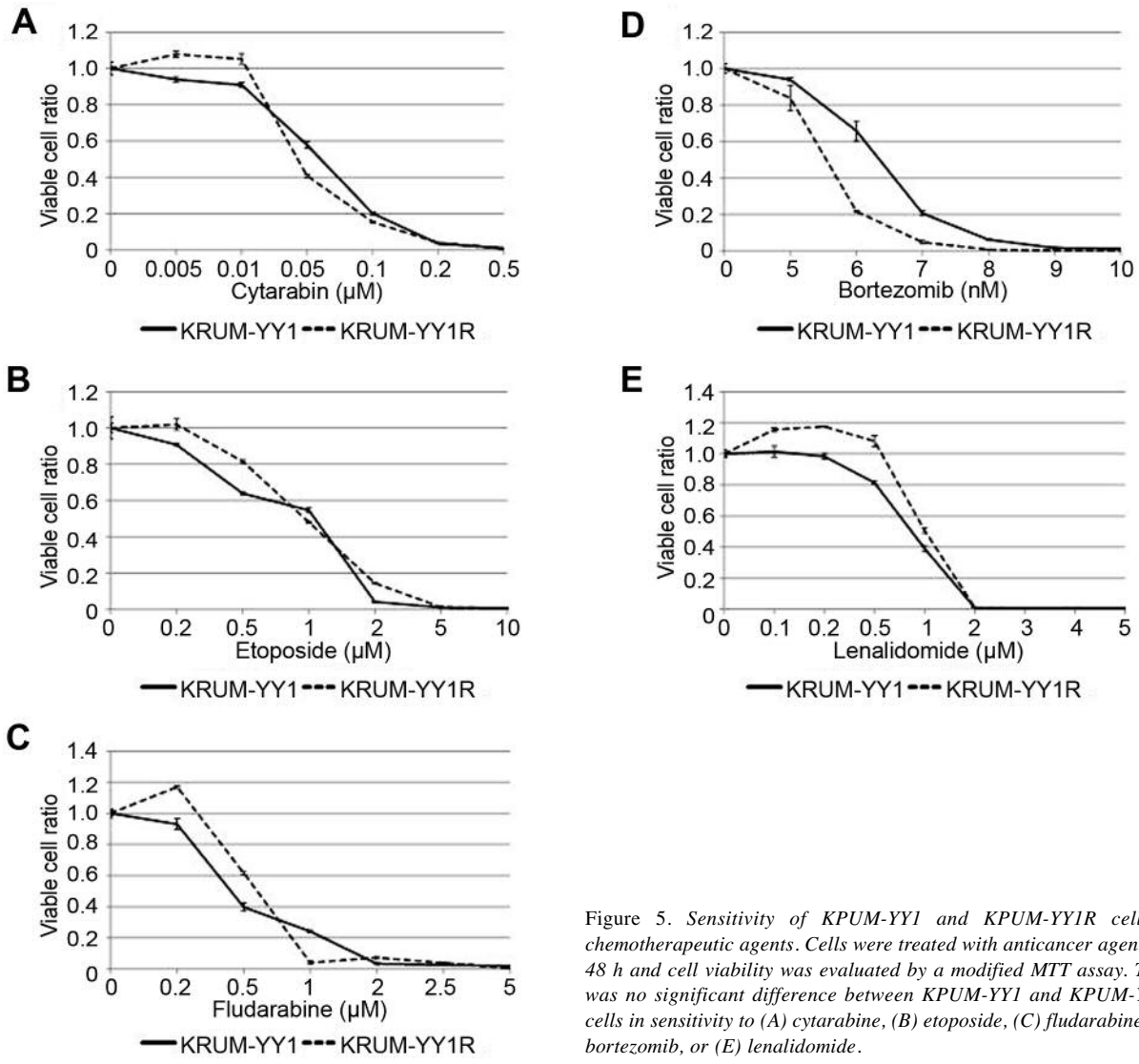

Figure 5. *Sensitivity of KPUM-YY1 and KPUM-YY1R cells to chemotherapeutic agents. Cells were treated with anticancer agents for 48 h and cell viability was evaluated by a modified MTT assay. There was no significant difference between KPUM-YY1 and KPUM-YY1R cells in sensitivity to (A) cytarabine, (B) etoposide, (C) fludarabine, (D) bortezomib, or (E) lenalidomide.*

alkylating agents or anthracyclines. Basic glutathione levels and GST activity showed no correlation to sensitivity to BH in previous studies (20, 32, 33), but we found that inhibition of GSTs partly restored the sensitivity to BH in KPUM-YY1R cells. Thus, we speculate that sensitivity and resistance to BH is associated with the GST level or another effect of GST that is cell- or tumor-dependent. There is no clinically available inhibitor for ABCB1 or GST, which suggests the importance of an appropriate clinical sequence of therapeutic agents for MCL. That is, once resistance to BH is acquired, use of agents with effects unrelated to P-gp or GST might be most rational.

There are some limitations to this study. We first established a parental KPUM-YY1 cell line from a patient with MCL who developed clinical resistance to BH, and it might have been expected that this cell line should also be resistant to BH. However, KPUM-YY1 cells retain sensitivity to various anticancer agents, including BH, in *in vitro* experiments. The precise reason for this discrepancy is unclear, but we speculate that there are additional mechanisms other than the ABCB1 and GST pathways for clinical resistance to BH *in vivo*, such as a protective effect of the tumor microenvironment or dysregulation of the pharmacological metabolism. It is also conceivable that the clonal tide during treatment may cause the discrepancy. Given that the patient was first sensitive to BH and then acquired clinical resistance, the BH-resistant clone might have become predominant after long exposure to BH in development of clinical resistance, but there could still be a small residual population of BH-sensitive clones, and the parental KPUM-

YY1 cells could have been mostly derived from this population. Regardless, the results showed involvement of ABCB1 and GST family proteins in acquisition of BH-resistance and secondary multidrug resistance in a MCL-derived cell line, but unknown mechanisms may also account for clinical resistance to BH. It will be important to investigate the clinical role of upregulation of ABCB1 or GST family proteins in MCL in a future study.

In conclusion, this study, for the first time, showed that common multidrug resistance mechanisms, such as ABCB1 and GSTs, underlie resistance to BH, and that acquisition of BH resistance potentially leads to multidrug resistance in MCL. The newly-developed KPUM-YY1 and KPUM-YY1R cells allowed identification of multiple mechanisms underlying BH activity and resistance, and may facilitate development of a strategy that overcomes treatment refractoriness in MCL.

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