1p31, 7q21 and 18q21 chromosomal aberrations and candidate genes in acquired vinblastine resistance of human cervical carcinoma KB cells

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Abstract. Vinblastine (VBL) is used to treat certain kinds of cancer including Hodgkin's lymphoma, lung cancer, breast cancer, testicular cancer and cervical carcinoma. However, the rapid development of resistance during therapy remains a major clinical challenge. In order to reverse cancer cell resistance, the goal of this study was to find differentially expressed genes and chromosomal alterations in multidrug resistant (MDR) KB-v1 cells, further to probe the relationship between drug resistance and differential genes, and chromosomal changes in MDR cancer cells. Comparative genomic hybridization (CGH) analysis of MDR KB-v1 and their parental KB-3-1 cells revealed chromosomal changes; microarray-based expression profiling was carried out by comparing the gene expressions of MDR KB-v1 cells and KB-3-1 cells. We have identified 3 chromosomal gains in regions of 1p31, 7q21 and 18q21 in MDR cells and 10 genes (*CYR61*, *UGTREL7*, *MBD1*, *NARS*, *ATP5A1*, *ABCB1*, *ABCB4*, *PEG10*, *MCM7*, *SERPINE1*) contained in these regions were also up-regulated in MDR KB-v1 cells. Forty-nine genes were down-regulated when KB-v1 cells were subjected to lower dose or depletion of the drug. We have confirmed some gene expression changes by reverse transcription-polymerase chain reaction and Northern blots. These are the first data describing the relationship of 1p31 and 18q21 chromosomal aberrations and candidate genes in acquired vinblastine-resistance. This study also demonstrates that the combination of CGH and cDNA microarray is a very useful tool to detect drug resistant targets in cancer treatment.

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Introduction

Drug resistance is the major obstacle to be overcome during the systemic therapy of cancer. The terms of drug resistance and sensitivity are relative conditions that must be defined with respect to some standard reference frames. Once a drug has achieved a critical threshold, it will interact with a range of cellular macromolecules. These genetic changes result in the development and progression of cancer and appear to be induced to multidrug resistance. Mechanisms implicated in cellular resistance include overexpression of the multidrug resistance gene (MDR1) (1), multidrug resistance-associated protein (MRP) (2); and anti-apoptosis, such as increasing tolerance of DNA damage and DNA repair (3). However, different cancer cells have their different cellular resistant mechanism.

Human cervical carcinoma KB (oral carcinoma) cells, used for selection of the multidrug resistant (MDR) cell lines, represent a subclone of human HeLa cells. These cells were chosen for the study of MDR because they are highly sensitive to most chemotherapeutic drugs. MDR derivatives of KB cells were obtained by selection with vinblastine, colchicine, or adriamycin, which is associated with multidrug resistance (4). KB-v1 cell is a derivation of the MDR KB cell lines. Its parent cell line was KB-3-1. KB-v1 cells were maintained with 1 μg/ml vinblastine for drug resistance. We have found that the phenotype of the highly resistant lines was unstable and most of the resistance was lost by growing the cells in the absence of the drugs for 3-6 weeks.

The mechanisms of drug resistance in cancer cells need to be thoroughly studied at the molecular level. Several lines of evidence have indicated that MDR in the highly resistant variants of KB cells was likely to be mediated by gene amplification. Comparative genomic hybridization (CGH) developed in 1992 has been utilized to identify DNA copy number abnormalities in various kind of cancers (5). In a typical CGH measurement, two genomes can be compared simultaneously, the total genomic DNA is isolated from test

and reference cell populations. The metaphase chromosomes were used for the representation of the genome and the location of copy number variations between test and reference genomic DNA was mapped to the physical position on the chromosomes (6). DNA amplification is also an important mechanism that allows cancer cells to increase expression of drug resistant related genes (7). CGH copy number profiles may facilitate identification of important new drug resistant genes located at the hotpots of the chromosomal alterations.

cDNA microarrays technique is a high-capacity system, which was developed to monitor the expression of many genes in a single experiment (8,9). cDNA microarray has been used to examine the development of drug resistance in cancer by comparing the gene expressions of MDR cancer cells and their parental cells (10-13). We have used statistical analysis of microarray data with Partek software. Partek Genomics Solution is a comprehensive suite of advanced statistics and interactive data visualization specifically designed to reliably extract biological signals from noise. It supports a complete workflow including convenient data access tools, identification and annotation of important drug resistant biomarkers. These analyses ultimately may enable us to use the signature expression profiles of drug resistance to predict response to drugs and to carry on chemotherapy or gene therapy.

In this study, we report the identification of genes associated with the vinblastine-resistant phenotype based on a different approach designed, advance technologies and software in MDR cells maintained with different doses of drug and comparing the expression patterns and copy number between MDR KB-v1 and its parental cell line.

Materials and methods

Cells. The KB cell line, KB-3-1 was purchased from the American Type Culture Collection. KB-v1 cell line is a derivation of the MDR KB cell lines, whose parent cell line was a KB subline designated KB-3-1. KB-v1 cells were obtained by selecting with vinblastine (VBL), which KB-3-1 was developed by selecting with vinblastine (starting at 0.2 μg/ml and ending up with 1 μg/ml). KB-3-1 and KB-v1 cells were cultured in DMEM media supplemented with 10% heat-inactivated fetal calf serum and antibiotics, penicillin and streptomycin.

Cytotoxicity assay. For the SRB (sulforhodamine B) assay standard procedures were used (14). KB cells were plated in 96-well plates (10^4 cells/well) and grown overnight at 37°C in a 5% CO_2 incubator. Test compounds (vinblastine or doxorubicin) were then added to the wells to achieve a final concentration ranging from 10^{-6} to 10^{-4} M. Control wells were prepared by adding 100 μl culture medium. The plates were incubated at 37°C in a 5% CO_2 incubator for 72 h. After adding 50 μl of 50% trichloroacetic acid (TCA) for 10 min at room temperature, the plates were then put at 4°C for 4 h and the supernate were removed, washed 5 times with 3D H_2O. Then, 35 μl 0.4% (wt/vol) SRB was added to each well for 30 min. Unbound dye was removed by four washes with 1% acetic acid. The protein-bound dye was extracted with 10 mM Tris base at a wavelength of 515 nm. The IC_{50} was determined from the plots of % viability vs. dose of compound added.

Cell cycle analysis. To determine cell cycle distribution, $5x10^5$ KB-3-1 and KB-v1 cells were plated in 60-mm dishes, treated with 0.2 μg/ml of vinblastine. Cells were then collected by trypsinization, fixed in 95% ethanol, washed in 1% BSA-PBS, resuspended in 1 μg/ml of RNase and 50 μg/ml of propidium iodide (PI), incubated for 30 min in the dark at 37°C, and analyzed by flow cytometry using a FACSCalibur. The data were analyzed using the ModFit DNA analysis program.

CGH. CGH was performed essentially as described previously (5,15). The genomic DNA was prepared from KB cell lines, using DNA isolation Kit (Boehringer Mannheim Corp., IN, USA). DNA was labeled by nick translation using direct Spectrum green (test) and Spectrum red (reference) conjugated with dUTP (Vysis, Downers Grove, IL). Target metaphase spreads were prepared from PHA-stimulated peripheral blood lymphocytes from a healthy donor. Slides were dehydrated in 70, 80 and 95% ethnol; denatured in 70% formamide/2X SSC at 70-72°C for 2 min; and further dehydrated in ice-cold ethnol. For each hybridization, 1 μg labeled test DNA, 1 μg reference DNA and 12.5 μg Cot-1 DNA were mixed and ethnol-precipitated. DNA was resuspended in 14 μl hybridization mix containing 50% formamide, 2X SSC and 10% dextran sulfate, denatured at 70°C for 5 min and applied to denatured metaphase spreads. Slides were incubated at 37°C in moist chamber for 2 days. Standard post-hybridization washes were performed. Slides were counterstained with DAPI and mounted with antifading solution (Vectashied; Vector, Burlingame, CA).

Digital image analysis. Digital image analysis was performed as previous described (15). CGH hybridizations were analyzed using a digital image analysis system containing a Zeiss Axioplan 2 microscope equipped with a Sensys cooled-charged device camera (Photometrics Ltd., Tucson, AZ, USA). Three images of each metaphase cell were captured using filter wheel-mounted, single band excitation Rhodamine, FITC and DAPI filters. The image analysis was carried out using the Quips CGH program (Vysis). Five metaphase cells were analyzed to generate fluorescence ratio profiles in each case. Interpretation of the profiles was performed according to the program guidelines. The thresholds used for the interpretation of gains and losses of a DNA sequence copy number was defined as a tumor/reference ratio 1.25 or 0.75, respectively, with both the standard and the reverse hybridization methods. High-copy-number amplification of a chromosomal region was defined as tumor/reference or KB-v1/KB-3-1 ratio 1.50.

cDNA microarrays experiments. The cDNA microarrays consisted of a total of 9982 known genes and EST clones from Incyte Cloneset. PCR products from these clones were prepared and spotted on glass slide according to standard procedures (16). RNAs were extracted from the cells using TRIzol reagent (Life Technologies, Inc.), as specified by the manufacturer. Fluorescent-labeled cDNA with Cy3 or Cy5

Table I. Cytotoxicity studies on KB cells by SRB assay (IC_{50}).

Drug	KB-3-1 IC_{50} (μM)	MDR KB-v1 IC_{50} (μM)	Resistance index[a]
Vinblastine	6.77×10^{-4}	≥ 1.1	≥ 1625
Doxorubicin	0.03	≥ 1	≥ 33

[a]Resistance index, ratio between the IC_{50} value of MDR KB-v1 and parental KB-3-1 cells.

(Amersham Pharmacia) was synthesized from 100 μg of total-RNA by oligo(dT)-primed polymerization using SuperScript II reverse transcriptase (Life Technologies, Inc.) as described. In each hybridization, we used the same reference RNA, which was extracted from KB-3-1, labeled with Cy3-dUTP and the test line KB-v1 with Cy5-dUTP. The two cDNA pools were mixed and applied to the array in a hybridization mixture containing 3.5X SSC, 0.3% SDS and 10 μg of yeast tRNA. Hybridization took place under a glass coverslip in a humidified 65°C hybridization chamber for 14-16 h. The slides were washed, dried and scanned by using ScanArray 4000.

Microarray data analysis. Average signal intensity and local background measurements were obtained for each spot on the array by using Scanalyze software. Local background was subtracted from the value of each spot on the array. Spots were considered negative and were eliminated from any further analysis if the values for both channels were less than a threshold value, which was defined as one SD above average value of negative control spots on the array. The two channels were normalized with respect to the median values for the remaining set of the array. The Cy5/Cy3 fluorescence ratios were calculated from the normalized values. Statistical analysis of gene expression microarray data was carried on by the software of Partek Genomics Solution.

Northern blotting. Total cellular RNA was isolated as described above, run on 1% agarose gel (15 μg per gel lane) containing 2.2 M formaldehyde, and transferred to a nitro-cellulose membrane. Membrane was dried at room temperature, and RNA was cross-linked by UV irradiation in a Stratalinker (Stratagene). Hybridization was performed in ExpressHyb Hybridization buffer (Clontech) containing 1×10^6 cpm/ml of ^{32}P-labeled cDNA probe at 65°C for 2 h. Blots were washed in 2X SSC and 0.1% SDS for 20 min, followed by a wash at 50°C in 0.2X SSC and 0.1% SDS for 20 min.

Semi-quantitative RT-PCR. The semi-quantitative analysis of mRNA expression levels of ABCB1, EST AI916259, SGK, ID1 in MDR KB-v1 maintained in 1 μg/ml VBL were evaluated by RT-PCR. It showed that the band intensity increased linearly with the amount of mRNA. ß-actin was used to normalize the RNA quantity in each sample.

Results

Cytotoxicity studies on KB cells. Resistant KB cells (KB-v1) were derived by treating the cells with stepwise increasing concentrations of vinblastine, starting at 0.2 μg/ml and ending up at 1 μg/ml vinblastine. We tested the cytotoxicity of vinblastine and doxorubicin using SRB assay in highly multidrug-resistant KB-v1 cells, as well as in the drug-sensitive parental KB-3-1 cells. KB-v1 cells proved to be highly resistant to vinblastine (VBL) and doxorubicin (Dox) at the lethal dose (IC_{50}) values of >1.1 and 1 μM, respectively (Table I). The flow cytometry assay provides information regarding cell cycle based on analysis of their DNA content. The DNA content of vinblastine-treated KB-3-1 cell and MDR KB-v1 was examined by propidium iodide staining and flow cytometry. At 12 h post-treatment with 0.2 μg/ml of vinblastine, KB-3-1 cells were arrested predominately at G2/M phase of the cell cycle and G1 phase was apparently decreased (Fig. 1). The result indicated that KB-3-1 arrested
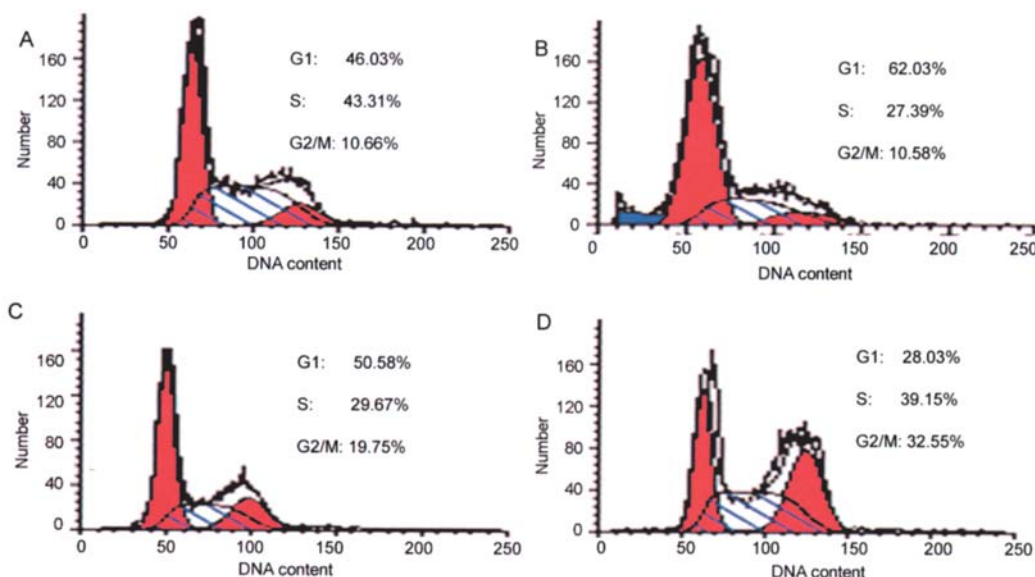

Figure 1. Effect of VBL on cellular DNA content. KB-3-1 and KB-v1 cells untreated and treated with VBL for 12 h. Cells were subjected to DNA content analysis by flow cytometry with PI staining. (A) KB-3-1 cells (untreated). (B) KB-v1 cells (1 μg/ml VBL). (C) KB-3-1 cells (0.2 μg/ml VBL, 30 min). (D) KB-3-1 cells (0.2 μg/ml VBL, 12 h).
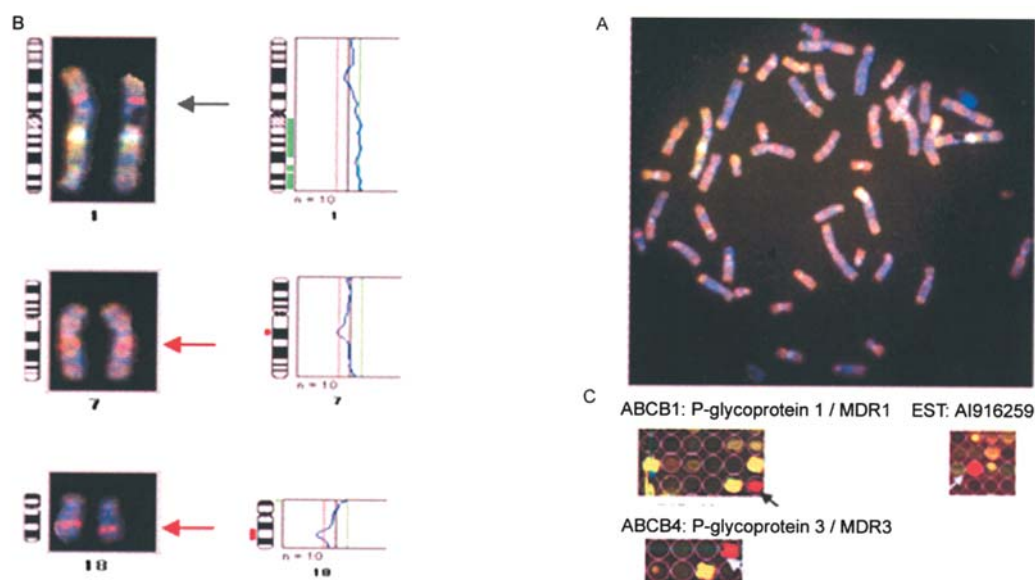
Figure 2. CGH and microarray for KB cell lines. (A) A representative image of CGH: MDR KB-v1 to KB-3-1 [KB-v1 labeled (spectrum red)/KB-3-1 labelled (spectrum green)]. (B) CGH analysis displays MDR KB-v1 cells have 3 chromosomal gains (1p31-1p22, 7q21 and 18q21) compared with its parental cell line (KB-3-1). (C) Microarray image of MDR1 genes. (KB-v1 labeled Cy5-dUTP/KB-3-1 labeled Cy3-dUTP; red spots showed MDR1 genes were overexpressed in MDR KB-v1 cells).

at G2/M in response to vinblastine, but MDR KB-v1 did not arrest at G2/M maintained in 1 μg/ml VBL. These results show that MDR KB-v1 is more resistant to vinblastine than its parental cell line KB-3-1.

CGH studies on chromosomal gains, loss and amplifications. Compared to normal reference, 7 and 13 genetic changes were found in KB-3-1, MDR KB-v1, respectively, including 4 losses and 3 gains in KB-3-1, 3 losses and 10 gains in MDR KB-v1. Fig. 2 and Table II demonstrate that some genetic imbalances are present in KB-3-1 and MDR KB-v1. We found that in chromosomal regions 1q12-1q21, 5p13-5p15, 5q32-5q34, both MDR KB-v1 and its parental KB-3-1 cells showed chromosomal gains, whereas at 9p11, 9q12-9q13, Y chromosome, mutual losses of genetic material were detected in the two KB cell lines by CGH. However, 1p31-1p22, 7q21, 17p13, 17q24, 18q21, Xp22, Xq27 showed chromosomal gains only in MDR KB-v1 and 15p13-15p12 showed chromosomal losses only in KB-3-1. In further studies, 6 genetic changes were also found in MDR KB-v1 cell line, including 3 losses (1q12-1q21, 9q12-9q13 and Y chromosome) and 3 gains (1p31-1p22, 7q21 and 18q21) compared with its parental KB-3-1 cell line. One example of CGH ratio profiles is shown in Fig. 2.

Gene expression profiling in KB resistant cells and CGH analysis is consistent with gene expression profiles. Hybridization results of microarrays with Cy3-labeled cDNA probe from KB-3-1 cells and Cy5-labeled MDR KB-v1 demonstrated that, ~11.44% of the cDNAs exhibit more than a 1.5-fold expression level change. It is remarkable that drug transport, cell survival, cytoskeleton, tumor associated antigen, signal transduction and transcription, and the regulatory network of lipid metabolism play a role in acquired resistance to vinblastine (Table III). CGH with genomic DNA prepared

from MDR KB-v1 as probe successfully identified genomic gains and/or losses in chromosomal regions coding putative genes associated with multidrug resistance. In the 3 chromosomal gains, 10 genes such as CYR61, UGTREL7, MBD1, NARS, ATP5A1, ABCB1, ABCB4, PEG10, MCM7, SERPINE1 were up-regulated in MDR KB-v1 cells using cDNA microarray analysis (Table IV). These genes included various members of families of ABC transporters, signal transduction, cytoskeleton proteins and metabolism.

Drug-resistance related genes were differentially expressed depending on drug concentration. Results of analysis of variance (ANOVA) by Partek Genomics Solution showed that the gene expression profiles of MDR KB-v1 were significantly different among three drug concentrations (0, 0.2 and 1 μg/ml VBL). By comparison of gene expression profiles in the drug-resistant and parental cells and at a normal cut-off value >1.5-fold, changes in the transcription levels were identified, 1467 genes/ESTs were differently expressed in MDR KB-v1 cells maintained in 1 μg/ml VBL, compared to its parental KB-3-1 cell line. We have also found that 379 genes/ESTs were differently expressed in the three drug concentrations of VBL in MDR KB-v1 cells (Fig. 3). Genes related to drug resistance were down-regulated in the present of low drug concentration or in the absence of drug. Table III shows that many genes related in drug resistance in MDR KB-v1 were down-regulated when the KB-v1 is absent of VBL compared to in 1 or 0.2 μg/ml VBL. Those genes were involved in ABC transporters (ABCB1, ABCA3 and ABCB2), signal transduction (SGK, STK12, STK15, SH3BP5, G3BP, PABPC4 and GEMIN4), inhibition of apoptosis (CRMP1, DAD1, DDB2, ID1 and UGT2B7), and lipid metabolism (ACAT1, LRP8 and HDLBP). Additional genes were keratin genes, melanoma antigen family and proteasome subunits.

Table. Chromosomal target regions and putative genes in multidrug resistant KB-v1 and its parental cell line.

Chromosomal target regions		Putative target genes
KB-3-1/normal	1q12-1q21 (gain)	TAGLN2 (1q21-q25), EFNA1 (1q21-q22), FDPS (1q21.2), ENSA (1q21.1), FLJ10687 (1q21.1), ILF2 (1q21.1), SH2D2A (1q21), PMF1 (1q12), TXNIP (1q12), RBM8A (1q12)
	5p13-5p15 (gain)	DAP (5p15.2), ENDOFIN (5p15.2-q14.3)
	5q32-5q34 (gain)	H2AFY (5q31.3-q32), SLC6A7 (5q31-q32), ATOX1 (5q32), TTC1 (5q32-q33.2), G3BP (5q33.1)
	9p11 (lost)	ND
	9q12-q13 (lost)	ND
	15p13-15p12 (lost)	ND
	Y chromosome (lost)	RPS4Y (Yp11.3), EIF1AY (Yq11.223)
KB-v1/normal	1p31-1p22 (gain)	CTBS (1p22), GTF2B (1p22-p21), CYR61 (1p31-p22)
	1q12-1q21 (gain)	TAGLN2 (1q21-q25), EFNA1 (1q21-q22), FDPS (1q21.2), ENSA (1q21.1), FLJ10687 (1q21.1), ILF2 (1q21.1), SH2D2A (1q21), PMF1 (1q12), TXNIP (1q12), RBM8A (1q12)
	5p13-5p15 (gain)	DAP (5p15.2), ENDOFIN (5p15.2-q14.3)
	5q32-5q34 (gain)	H2AFY (5q31.3-q32), SLC6A7 (5q31-q32), ATOX1 (5q32), TTC1 (5q32-q33.2), G3BP (5q33.1)
	7q21 (gain)	ABCB1 (7q21.1), ABCB4 (7q21.1), ESTAI916259 (7q21.1), PEG10 (7q21), SERPINE1 (7q21.3-q22), MCM7 (7q21.3-q22.1)
	17p13 (gain)	MYO1C (17p13), GEMIN4 (17p13), EIF4A1 (17p13), PSMB6 (17p13), ITGAE (17p13), POLR2A (17p13.1), STK12 (17p13.1), PMI (17p13.1), HSA272196 (17p13.2), SPAG7 (17p13.3), ALOX15 (17p13.3), PAFAH1B1 (17p13.3)
	17q24 (gain)	FALZ (17q24), FLJ21347 (17q24.1)
	18q21 (gain)	BCL2 (18q21), MBD1 (18q21), NARS (18q21.2-q21.3), ATP5AI (18q12-q21), MAPK4 (18q12-q21)
	Xp22 (gain)	ND
	Xq27 (gain)	ND
	9p11 (lost)	ND
	9q12-q13 (lost)	ND
	Y chromosome (lost)	RPS4Y (Yp11.3), EIF1AY (Yq11.223)
KB-v1/KB-3-1	1p31-1p22 (gain)	CTBS (1p22), GTF2B (1p22-p21), CYR61 (1p31-p22)
	7q21 (gain)	ABCB1 (7q21.1), ABCB4 (7q21.1), SRI (7q21.1), ESTAI916259 (7q21.1), PEG10 (7q21), SERPINE1 (7q21.3-q22), MCM7 (7q21.3-q22.1)
	18q21 (gain)	BCL2 (18q21), MBD1 (18q21), NARS (18q21.2-q21.3), ATP5A1 (18q12-q21), MAPK4 (18q12-q21)
	1q12-q21 (lost)	TAGLN2 (1q21-q25), EFNA1 (1q21-q22), FDPS (1q21.2), ENSA (1q21.1), FLJ10687 (1q21.1), ILF2 (1q21.1), SH2D2A (1q21), PMF1 (1q12), TXNIP (1q12), RBM8A (1q12)
	9q12-9q13 (lost)	ND
	Y chromosome (lost)	RPS4Y (Yp11.3), EIF1AY (Yq11.223)

Northern blots and semi-quantitative RT-PCR for verification of the array hybridization data. Northern blot analysis of BCL2L1, PTEN, CLU, CGA, ENO3, KRT7 and ß-actin was performed on RNA prepared from KB-3-1 cells and KB-v1 cells, which were maintained in 0.2 µg/ml VBL.

These results demonstrated that BCL2L1, PTEN, CGA, CLU, ENO3 and KRT7 were overexpressed in KB-v1 compared to ß-actin as control (Fig. 4A).

The semi-quantitative analysis of mRNA expression levels of ABCB1, EST AI916259, SGK, ID1 in MDR KB-v1

Table III. The part of differently expressed genes detected by DNA microarray analysis.

Genes/ESTs	No drug	0.2 μg/ml VBL	1 μg/ml VBL	Gene name	Gene Bank no.
Drug transport					
P-glycoprotein 1/multiple drug resistance 1	1.153	1.370	4.531	ABCB1	NM000927
ESTs	6.737	8.113	31.36	ABCB1	AI916259
P-glycoprotein 3/multiple drug resistance 3	2.773	2.267	10.66	ABCB4	X06181
ATP-binding cassette 3	-2.25	-1.30	1.69	ABCA3	NM_001089
Transporter 1, ABC (ATP binding cassette)	-1.35	-1.25	1.603	TAP1	NM_000593
ATP-binding cassette 50	-1.49	1.318	1.463	ABCF1	NM_001090
ATP-binding cassette, sub-family B	1.550	1.629	1.253	ABCB6	AI653711
Solute carrier family 6	2.584	3.04	4.551	SLC6A7	AI986397
Solute carrier family 4, anion exchanger	1.223	-1.53	-2.31	SLC4A2	NM_000340
Solute carrier family 38, member 1	-4.083	-1.76	-2.83	SLC38A1	BF348345
Cell survival					
Defender against cell death 1	4.512	5.31	3.86	DAD1	AV714320
Collapsin response mediator protein 1	1.452	1.372	3.623	CRMP1	BF343909
Clusterin	3.571	5.439	3.506	CLU	BF525595
Inhibitor of DNA binding 1	-1.42	2.927	4.268	ID1	AL117381
UDP glycosyltransferase 2 family, polypeptide B7	-1.71	2.385	2.15	UGT2B7	BF689099
Damage-specific DNA binding protein 2 (48 kDa)	1.497	1.385	2.149	DDB2	NM_000107
Enolase 3, (ß, muscle)	1.094	2.448	1.503	ENO3	NM_001976
Inhibitor of growth family, member 1	1.666	-5.297	-2.275	ING1	AF181850
Tumor rejection antigen (gp96) 1	1.021	-1.06	-3.463	TRA1	AV655628
Thioredoxin interacting protein	-2.046	-8.274	-7.838	TXNIP	AU139227
Heat shock protein 75	-2.258	-2.679	-52.11	TRAP1	BE901723
Proteasome 26S subunit, non-ATPase, 11	-1.55	2.611	1.405	PSMD11	AB003102
Proteasome 26S subunit, ATPase, 5	1.163	2.279	2.037	PSMC5	AI568351
Proteasome 26S subunit, non-ATPase, 3	1.318	2.786	1.982	PSMD3	NM_002809
Ubiquitin specific protease 11	1.071	1.744	1.904	USP11	BG392606
Ubiquinol-cytochrome c reductase subunit	-1.17	2.033	2.104	UQCR	AW163002
Melanoma antigen					
Melanoma antigen, family D, 2	-1.31	2.279	2.672	MAGED2	BF969633
Melanoma antigen, family A, 12	-1.18	2.888	2.582	MAGEA12	L18877
Melanoma antigen, family A, 2	-1.72	2.218	2.396	MAGEA2	BG029985
Melanoma antigen, family A, 6	-1.45	1.958	1.671	MAGEA 6	NM_005363
CD63 antigen (melanoma 1 antigen)	1.175	1.464	1.944	CD63	BE907932
Cytoskeleton					
Keratin, hair, basic, 3	1.950	2.737	5.992	KRTHB3	NM_002282
Keratin 8	2.055	1.782	2.999	KRT 8	B100080
Keratin 17	1.019	1.395	2.60	KRT17	AI521362
Keratin 7	-2.21	2.273	2.51	KRT7	AA307373
Keratin 7	-2.14	2.235	2.493	KRT7	BI094014
Tropomyosin 4	-1.31	-1.05	2.242	TPM4	BC002827
Crystallin, α B	-1.51	1.109	2.552	CRYAB	BF727296
Selenoprotein W, 1	1.117	1.519	2.815	SEPW1	BG476052
Lectin, galactoside-binding, soluble	2.421	2.308	2.614	LGALS3BP	L13210
Membrane protein of cholinergic synaptic vesicles	1.150	2.262	3.193	VATI	BG763127
Signal transduction and transcription					
Glycoprotein hormones, α polypeptide	5.221	5.293	5.247	CGA	BG620346

Continued.

Genes/ESTs	No drug	0.2 µg/ml VBL	1 µg/ml VBL	Gene name	Gene Bank no.
Serum/glucocorticoid regulated kinase	-1.22	3.636	4.705	SGK	BG756781
Nuclear body protein Sp140	5.370	4.661	5.590	Sp140	NM_007237
Insulin-like growth factor binding protein 6	1.189	2.97	3.227	IGFBP6	BG326454
Adhesion regulating molecule 1	-1.63	-1.05	2.145	ADRM1	BF033463
Serine/threonine kinase 15	-1.13	1.896	2.1	STK15	NM_003600
Serine/threonine kinase 12	-1.10	1.989	1.568	STK12	BG616089
CD27-binding (Siva) protein	1.108	1.559	3.086	SIVA	AI267883
Inhibitor of DNA binding 1	-1.42	2.927	4.268	ID1	AL117381
SH3-domain binding protein 5	-1.56	1.437	6.296	SH3BP5	BG030766
Ras-GTPase-activating protein	-1.04	1.108	1.914	G3BP	BG252290
SH2 domain protein 2A	-2.398	-3.46	-4.652	SH2D2A	NM_003975
Poly(A) binding protein, cytoplasmic 4	-2.60	2.468	1.906	PABPC4	BG392606
Poly(A) binding protein-interacting protein 1	-4.386	-1.56	-2.206	PAIP1	AL548163
Activating transcription factor 1	-2.406	-1.814	-2.843	ATF1	AI004969
Translocated promoter region	-1.08	-1.587	-3.28	TPR	AI765790
CCAAT/enhancer binding protein (C/EBP)	-1.43	-2.12	-3.919	CEBPB	W39546
Vascular endothelial growth factor	-1.667	-12.343	-2.288	VEGF	AI085744
Lipid/cholesterol metabolism					
High density lipoprotein binding protein	-1.93	1.772	2.758	HDLBP	NM_005336
Phospholipid scramblase 3	1.155	2.77	2.296	PLSCR3	AF159442
Acetyl-Coenzyme A acetyltransferase 1	2.242	1.818	2.068	ACAT1	AI683154
3-hydroxy-3methylglutaryl-Coenzyme A synthase1	1.340	2.928	2.816	HMGCS1	AW117731
Cholinergic receptor, nicotinic, α polypeptide 3	-1.07	-2.928	-2.888	CHRNA3	AA927784
Low density lipoprotein receptor-related protein 8	-1.758	-1.962	-2.895	LRP8	BC006443
Other metabolic genes					
UDP glycosyltransferase 2 family, polypeptide B7	-1.71	2.385	2.15	UGT2B7	BF689099
Prolylcarboxypeptidase (angiotensinase C)	-1.32	1.386	2.135	PRCP	AL540876
Protein geranylgeranyltransferase type I	-1.51	-1.64	2.612	PGGT1B	AA481712
Thiopurine S-methyltransferase	1.202	-1.35	-3.212	TPMT	AA974892
Deoxyribonuclease I	-1.07	-1.37	-2.143	DNASE1	AI802091
Deoxycytidine kinase	1.241	-2.951	-2.27	DCK	AI743955
Tyrosyl-tRNA synthetase	-1.776	-1.653	-2.887	YARS	NM_003680
Dual specificity phosphatase 11	1.251	-3.04	-2.87	DUSP11	AI478561
Asparagine synthetase	-2.622	-3.798	-3.187	ASNS	NM_001673
Dihydrofolate reductase	-1.41	-3.436	-3.376	DHFR	NM_000791
Peptidylprolyl isomerase (cyclophilin)-like 3	-1.02	-1.29	-3.529	PPIL3	AW971618
Selenophosphate synthetase 2	-1.47	-1.707	-3.733	SPS2	AA604652
Dual-specificity tyrosine-(Y)-phosphorylation	1.356	-1.09	-3.753	DYRK1A	D86550
Procollagen-lysine, 2-oxoglutarate 5-dioxygenase	-1.996	-1.27	-3.821	PLOD	AV711206
Alkaline phosphatase, placental (Regan isozyme)	-1.15	-2.083	-4.271	ALPPL2	BF807922

The data are presented as the average (Avg) of three independent gene expression measurements expressed as a fraction of the total hybridization signal on each DNA microarray.

maintained in 1 µg/ml VBL were evaluated by RT-PCR. The result showed that the band intensity increased linearly with the amount of mRNA. ß-actin was used to normalize the RNA quantity in each sample (Fig. 4B). Northern blots and semi-quantitative RT-PCR analysis of highly expressed genes verified the array hybridization data.

Table IV. CGH and cDNA microarray analysis of chromosomal target regions and up-regulated genes in MDR KB-v1 cells.

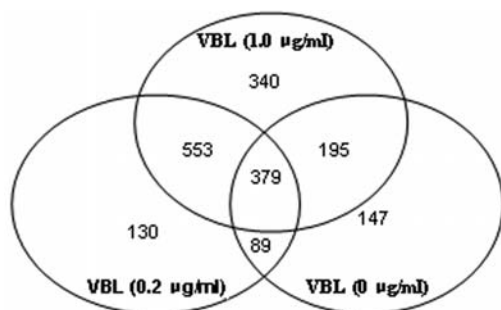| Gene symbol	Chromosome	KB-v1 to 3-1	Gene	Accession no.
CYR61	1p31-p22	3.196	Cysteine-rich, angiogenic inducer, 61	Y12084
UGTREL7	1p32-p31	3.149	UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter	D87449
MBD1	18q21	1.520	Methyl-CpG binding domain protein 1	BE747161
NARS	18q21.2-q21.3	4.047	Asparaginyl-tRNA synthetase	BG706529
ATP5A1	18q12-q21	1.800	ATP synthase, H$^+$ transporting, mitochondrial F1 complex	AU143446
ABCB1	7q21.1	4.531	ATP-binding cassette, sub-family B (MDR/TAP), member 1	NM000927
ABCB1	7q21.1	31.361	ATP-binding cassette, sub-family B (MDR/TAP), member 1	AI916259
ABCB4	7q21.1	10.660	ATP-binding cassette, sub-family B (MDR/TAP), member 4	X06181
PEG10	7q21	1.915	Paternally expressed 10	BG619874
MCM7	7q21.3-q22.1	1.959	Minichromosome maintenance deficient (S. cerevisiae) 7	BG683250
SERPINE1	7q21.3-q22	1.553	Plasminogen activator inhibitor, type I	BE812315

Figure 3. Differently expressed genes in MDR KB-v1 maintained in different drug concentration of VBL. Fold change values were calculated for all genes in MDR KB-v1 compared to its parental KB-3-1 cells. We identified 1467 genes/ESTs in the MDR KB-v1 maintained in 1 µg/ml VBL that had a >1.5-fold difference in expression, and 379 genes/ESTs were commonly differentially expressed in MDR KB-v1 maintained in 0, 0.2 and 1 µg/ml VBL.

Discussion

Drug resistance occurs in cancer through various mechanisms. In this study, global expression profiles and genomic alterations in MDR KB-v1 cells were comprehensively analyzed using cDNA microarray and CGH.

CGH has typically been applied to identify chromosomal aberrations in KB cancer cells DNA relative to normal genomic DNA. There were some common chromosomal alteration in both KB cancer cell lines. The regions 1q12-1q21, 5p13-5p15, 5q32-5q34 exhibited chromosomal gains in both cell clones. Common genetic losses were found in the regions 9p11, 9q12-9q13 and Y chromosome. These common chromosomal aberrations in KB cells identified the genome changes in human cervical carcinoma.

CGH analysis of MDR KB-v1 cell line identified gains of 1p31-1p22, 7q21 and 18q21 and losses of 1q12-1q21,
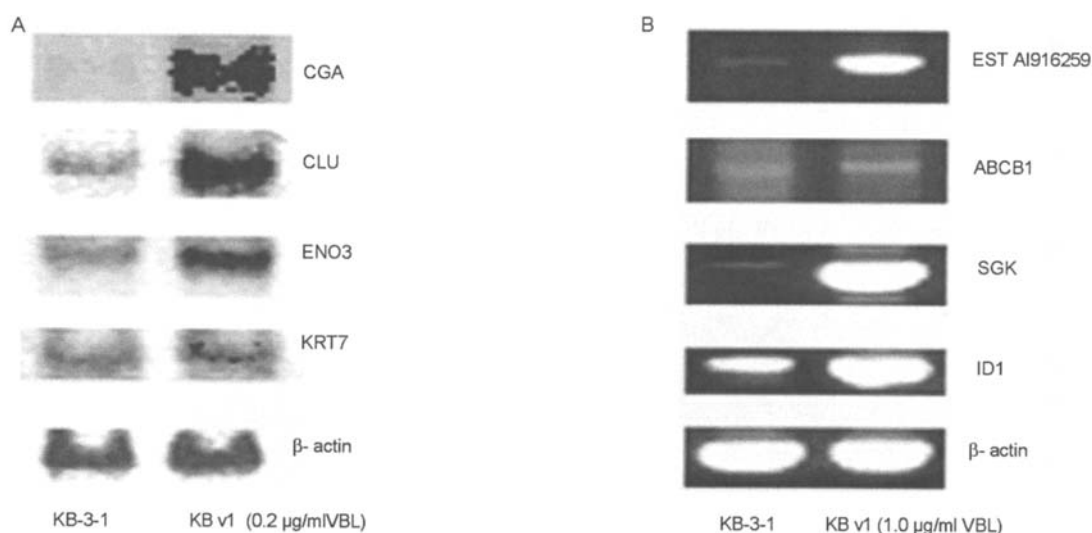

Figure 4. (A) Northern blots and (B) semi-quantitative *RT-PCR* analysis of *CGA*, *CLU*, *ENO3*, *KRT7*, *ABCB1*, *ID1*, *SGK* and *ESTAI916259*, and ß-actin was used to normalize the *RNA* quantity in each sample. These results verify the array hybridization data.

13 and Y chromosome as the most prominent markers by comparing with its parental cell line, KB-3-1 cells. It is well recognized that resistant cells with genetic alterations possessing a growth or survival advantage would lead to clonal expansion. Chromosomal 7q21 has also been reported in previous publications of MDR cancers (17-19). This is particularly helpful for MDR candidate evaluation. We determined correlations between chromosomal aberration and up- or down-regulation for 27 genes (Table II). The microarray analysis revealed that genes in the 7q21.1 chromosomal region, ABCB1, ABCB4, EST AI916259, were constitutively overexpressed in MDR KB-v1 cells. Other ABC transporters such as TAP1 (transporter 1), ABCA3, ABCB6 and ABCF1 were also up-regulated in MDR KB-v1 cells (Table III). These ATP binding cassette (ABC) transporters, localized in the cell membrane, caused drug resistance by extruding a variety of chemotherapeutic agents from the tumor cells. ABCB1, EST AI916259 and EST AA994037 were further verified highly expressed in MDR KB-v1 with semi-quantitative RT-PCR (Fig. 4). Computational analysis by genome mapping indicated that EST AA994037, EST AI916259 belongs to the 3'-untranslated regions (UTR) of ABCB1. P-glycoprotein (P-gp) coded for by mdr-1 (ABCB1) is an ABC (ATP-binding cassette) transporter, which hydrolyses ATP and extrudes cytotoxic drugs from mammalian cells.

P-gp association with the actin cytoskeleton was the key in conferring to human lymphoid cells a multidrug resistance phenotype (20). We found the tissue remodeling proteins such as KRTHB3, KRT7, KRT8, KRT17, TPM4, CRYAB, SEPW1, LGALS3BP and VATI were up-regulated in resistant cells (Table III). These showed that the intracellular vesicular transport of many compounds is partly controlled by cytoskeletal filaments.

The amplified region of 18q21 may contain more than one important gene, including BCL2 (18q21), MBD1 (18q21), NARS (18q21.2-q21.3), ATP5A1 (18q12-q21) and MAPK4 (18q12-q21). Up-regulation of Bcl2 protein expression might be one of the mechanisms of drug resistance in some cancer cells (21-23). ATP5A1 and ATP5C1 were also over-expressed in other resistant leukaemic cells (24). MBD1 was overexpressed in MDR KB-v1 cells. DNA methylation is the most common epigenetic alteration in tumor genomes and might result in transcriptional repression of tumor suppressor genes. Specific methylation patterns are involved in the development of therapy resistance of tumor cells (25).

The amplified region of 1p31-1p22 contained CTBS (1p22), GTF2B (1p22-p21), and CYR61 (1p31-p22). CYR61 (CCN1) is an extracellular matrix-associated protein of the CCN family. CYR61 functions through integrin-mediated pathways to promote cell adhesion, migration, and proliferation, and can also promote cell survival and tubule formation in human umbilical vein endothelial cells and induce E2-independence and antiestrogen-resistance (26,27). These overexpressed genes helped identifying the drug resistance mechanism involving in many pathways such as changing cell membrane permeability, degradation, modification, transportation of drug and increasing drug efflux.

In our cDNA microarray, we also identified several repressed genes, which mapped to the regions of loss defined by CGH analysis of MDR KB-v1, compared to KB-3-1 cell line. These included TXNIP at 1q12 and SH2D2A (SH2 domain protein 2A) at 1q21. SH2 domain containing proteins play a key role in the process of intracellular transmission of signaling events initiated at the cell surface (28). TXNIP was identified as a negative regulator of thioredoxin (TXN). TXN is induced by a variety of oxidative stimuli, including UV irradiation, inflammatory cytokines and chemical carcinogens and has been shown to play crucial roles in the regulation of cellular responses such as gene expression, cell proliferation and apoptosis (29).

Besides chromosomal changes in MDR cells, some transcription factors (such as ATF1, BZAP45, TPR and CEBPB) and replication factor (RFC4) were repressed in MDR KB-v1 cells. Activating transcription factor 1 (ATF1), CREB, and the cyclic AMP (cAMP) response element modulatory protein (CREM), which constitute a subfamily of the basic leucine zipper transcription factors, activate gene expression by binding as homo- or heterodimers to the cAMP response element in regulatory regions of target genes. Those genes down-regulated in MDR KB-v1 may act as negative regulators of stress response mediating cell survival by altering drug sensitivity. Interesting, we also found that ID1 (inhibitor of DNA binding1) was up-regulated in MDR KB-v1 cells. ID family of helix-loop-helix proteins are generally considered to be negative regulators of similar proteins, mostly transcriptional activators (30).

In conclusion, CGH analysis of the KB-3-1 and KB-v1 cell lines revealed 6 genetic changes in MDR KB-v1 cell line, including 3 losses (1q12-1q21, 9q12-9q13 and Y chromosome) and 3 gains (1p31-1p22, 7q21 and 18q21) compared with its parental cell line KB-3-1. We determined correlations between chromosomal aberration and up- or down-regulation for 27 genes. These genes included various members of families of ABC transporters, cell survival, signal transduction, cytoskeleton proteins and metabolism, which are promising MDR candidates.

Acknowledgements

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