Alteration in Copy Numbers of Genes as a Mechanism for Acquired Drug Resistance

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ABSTRACT

Chemoresistance is a major obstacle for successful treatment of cancer. To identify regions of the genome associated with acquired resistance to therapeutic drugs, we conducted molecular cytogenetic analyses of 23 cancer-cell lines, each resistant to either camptothecin, cisplatin, etoposide (VP-16), Adriamycin, or 1-beta-arabinofuranosylcytosine, although the parental tumor lines were not. Subtractive comparative genomic hybridization studies revealed regions of gain or loss in DNA-copy numbers that were characteristic of drug-resistant cell lines; i.e., differences from their drug-sensitive parental cell lines. Thirteen ATP-binding cassette (ABC) transporter genes [ABCA3, ABCB1 (MDR1), ABCB6, ABCB8, ABCB10, ABCB11, ABCC1 (MRP1), ABCC4, ABCC9, ABCD3, ABCD4, ABCE1, and ABCF2] were amplified among 19 of the resistant cell lines examined. Three genes encoding antiapoptotic BCL-2 proteins (BCL2L10, BCL2L2, and BCL2L1) were also amplified and consequently overexpressed in three of the derivative lines. Down-regulation of BCLXL2 with an antisense oligonucleotide sensitized a VP-16 resistant ovarian-cancer cell line (SKOV3/VP) to VP-16. A decrease in copy numbers of genes encoding deoxycytidine kinase, DNA topoisomerase I, and DNA topoisomerase II α reduced their expression levels in one cytotoxic arabinoside-resistant line, two of three camptothecin-resistant lines, and two of five VP-16-resistant cell lines, respectively. Our results indicated that changes in DNA-copy numbers of the genes mentioned can activate or down-regulate them in drug-resistant cell lines, and that such genomic alterations might be implicated in acquired chemoresistance.

INTRODUCTION

Resistance to chemotherapy agents is a major obstacle for successful treatment of cancer. Most cancers are intrinsically resistant to chemotherapy or become resistant after an initial partial response (1). Chemoresistance, whether intrinsic or acquired, is attributable to genetic or epigenetic processes taking place in neoplastic cells (2). Several explanations for drug resistance have been put forward: (a) decreased accumulation of drug within cells because of reduced inward transport or increased drug efflux; (b) enhanced inactivation or detoxification of the drug; (c) failure to convert the prodrug to its active form; (d) altered amounts or activities of target proteins; (e) enhanced capacity for DNA repair; and (f) increased resistance to apoptosis (1, 2). Multiple factors in that list appear to coexist in cancer cells. An increased understanding of mechanisms underlying drug resistance may lead to the development of more successful therapeutic protocols.

Gene amplification and consequent overexpression of ABCB1 (MDR1) or ABCC1 (MRP1) have been observed in a considerable number of drug-resistant cell lines (3). Previous comparative genomic hybridization (CGH) studies using cell lines or primary tumors have revealed other regions of chromosomal imbalance associated with acquired resistance (4–8). Regions involved in amplifications or deletions characteristic of drug-resistant cells are likely to contain additional genes, therefore unidentified, that contribute to chemoresistance.

In the study reported here we explored genomic alterations that might be associated with acquired chemoresistance, using 23 cell lines derived from human neoplasms, each of them resistant to at least one of five drugs including camptothecin (CPT), cisplatin (cDDP), etoposide (VP-16), Adriamycin, and cytosine arabinoside (Ara-C). We performed molecular cytogenetic studies including subtractive CGH with DNAs from corresponding pairs of resistant lines and drug-sensitive parental cells to disclose chromosomal regions that had gained or lost DNA during the acquisition of resistance. Our results provide evidence for alteration in gene-copy numbers as a mechanism for acquired chemoresistance.

MATERIALS AND METHODS

Cell Lines. The 23 drug-resistant cancer-cell lines selected for this study had been established from 10 drug-sensitive human cancer-derived cell lines (HT-29, A549, SK3, St-4, A2780, SKOV3, K47, T24, K562, and U937; Table 1). Three were resistant to CPT, 8 to cDDP, 10 to VP-16, 1 to Adriamycin, and 1 to Ara-C. HT-29/ADR, HT-29/ETP, and A2780/cDDP cells were established in the laboratory of Prof. Takashi Tsuru at the University of Tokyo; clones 21, 36, 41, and 49, derived from U937 cells, were established in the same laboratory. SK3/VP16, SKOV3/VP, K562/etop20, and K562/etop80 cells were a kind gift from Dr. Kiyohiro Nishikawa, Nippon Kayaku Co. (Tokyo, Japan). Other lines had been described previously (Table 1). CGH Analysis. CGH analyses were performed as described elsewhere (9). Shifts in CGH profiles were rated as gains or losses if they reached at least the respective 1.2 or 0.8 thresholds. Over-representations were considered to be high-level gains when the fluorescence ratios exceeded 1.5, as described elsewhere (9). Fluorescence in Situ Hybridization (FISH) Analysis. We performed FISH using as probes appropriate bacterial artificial chromosomes (BACs) and P1-artificial chromosomes as described previously (10). Each of BAC or P1-artificial chromosome probe was identified by gene name listed in Tables 2 and 3. We used RP11–35609 as the probe for HNF3α (10).

Northern Blot Analysis. Northern hybridizations were performed as described previously (10). Probes for this experiment consisted of two expressed sequence tags, IMAGE clone 179576 for ABCA3 and clone 2205297 for ABCC1 (MRP1), both purchased from Incyte Genomics (Palo Alto, CA). BCL2L2-specific probe was synthesized by PCR using a primer pair (Forward, 5′-TATAAGCTGAGGCAGAAGGG-3′; Reverse, 5′-TCAGCCTGTCTC- CACTGAT-3′) according to Kitamura et al. (11). GAPDH was used as a control for estimating loading differences on the blots (10). Hybridization signals were measured with a BAS-2500 image analyzer (Fuji Film, Tokyo, Japan).
Expression levels were quantified by normalizing the ratio of gene
mRNA levels were quantified using a real-time fluorescence detection method described elsewhere.

Caspase-3 activity was measured in the supernatant fraction (100 µg of protein) according to its proteolytic cleavage of the colorimetric substrate Ac-DEVD-pNA (Promega, Madison, WI) according to its proteolytic cleavage of the colorimetric substrate Ac-DEVD-pNA.

Table 2

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*CGH, comparative genomic hybridization; CPT, camptothecin; CDDP, cisplatin; ADM, Adriamycin; VP-16, etoposide; 3A-C, cytosome arabinoside.

Antisense Experiments. Antisense experiments were performed as described elsewhere (9). Briefly, two oligonucleotides containing phosphorothioate backbones (OPTs) were synthesized (Espeol Oligo Service Corporation, Tsukuba, Japan), AS, 5'-AGGATCCCCTCCACCATCCGA-3'; the antisense direction of BCL2L2 cDNA nucleotides 2374 to 2394; GenBank accession no. NM 004050), and SC, 5'-AGCCTACCACCTCCCCTAGAA-3' (the scramble control for AS). The AS- and SC-OPTs were delivered into cells using Oligofectamine (Invitrogen, Carlsbad, CA). For determination of mRNA levels, cells were harvested 24 h after transfection. For viability assays, cells were seeded in 96-well plates (2500 cells/well) the day before transfection; 24 h after introduction of 400 µl AS or SC into cells, VP-16 was added at final concentrations of 1, 3, or 10 µg/ml, and maintained for 48 h. Viable cells were counted by the colorimetric water-soluble tetrazolium salt assay (Cell-Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) 72 h after transfection.

RESULTS

Genomic Changes Specific to Drug-Resistant Cell Lines. An overview of the genetic changes detected by subtractive CGH in 23
were definitely amplified in 19 of the drug-resistant cell lines as the other 3 (ABCC6). The 48 known genes encoding ABC transporter proteins are widely distributed in the human genome (12). Chromosomal locations of 20 such genes showed gains of DNA in our drug-resistant cell lines. Among them we examined 17 of these 20 ABC transporter genes by FISH; no BAC or P1-artificial chromosome probes were available for the other 3 (ABCC6, ABCC7, and ABCC8).

FISH analyses revealed that genes encoding 13 ABC transporters were definitely amplified in 19 of the drug-resistant cell lines as compared with their parental lines (Table 2). In particular, ABCA3, ABCB1 (MDR1), and ABCC9 (SUR2) showed >2-fold increases in gene-copy numbers. In HT-29/ETP cells having a region of gain at 16p12-p13 (Table 1) by CGH, the number of FISH signals for ABCA3 (located at 16p13.3) and ABCC1 (MRP1; 16p13.1) were 7 and 5 respectively, whereas each of those genes yielded only 3 signals in parental (HT-29) cells (Fig. 1, A and B). These findings indicated that ABCA3 is more likely to be a target of the amplification mechanism than ABCC1. Indeed, we constructed the refined map of the amplicon by additional FISH, and defined the smallest region harboring ABCA3 between BACs RP11–334D3 and RP11–95P2 (containing markers D16S525 and SHGC-11838, respectively) distal to ABCC1 (Fig. 1f). SK3/VP16 cells produced 11 FISH signals for ABCC1, located at 7q21, in contrast with 3 in its parental (SK3) cells, suggesting that this gene was a probable target within the 7q21-q22 amplicon. SK3/VP16 and SKOV3/VP cell lines each showed 5 FISH signals for ABCC9, located at 12p12.1, whereas parental cells had only 2.

To determine whether gene amplification was consistently associated with up-regulation of its expression, we examined expression levels of ABCA3, ABCB1, ABCC1, and ABCC9 in drug-resistant cell lines and their parental cells. Northern blotting revealed that ABCA3 and ABCC1 were overexpressed in HT-29/ETP cells (ratios of 10.6 and 2.6, respectively) compared with HT-29 cells (Fig. 2a). This finding supported the notion that ABCA3 was a more probable target within the amplified region at 16p12–13 than ABCC1. Real-time quantitative reverse transcription-PCR analysis showed that SK3/VP16 cells exhibited a 3.3-fold increase in expression of ABCB1 than SK3 cells (data not shown). Expression of ABCC9 was up-regulated in SK3/VP16 cells with a ratio of 13.3 compared with SK3 cells; on the other hand, it was only minimally overexpressed in SKOV3/VP cells (ratio of 1.6) compared with SKOV3 cells (Fig. 2c).

Amplification and Overexpression of Genes Encoding Anti-apoptotic Proteins of the BCL2 Family. BCL2, BCL2L1 (BCL-XL), BCL2L2 (BCL-W), BCL2A1, MCL1, and BCL2L10 encode BCL-2 proteins that act as antiapoptotic regulators (13). Among these 6 genes, all except BCL2L1 (BCL-XL) were located in regions showing gains of DNA in drug-resistant cell lines. FISH analyses showed that BCL2L2, MCL1, and BCL2L10 were certainly amplified in SKOV3/VP, HT-29/CTP, and K562/AC cells respectively, compared with their parent cells (Table 2). Notably, in SKOV3/VP cells showing amplification at 14q11-q21, six FISH signals were evident for BCL2L2 (14q11.2-q12), in contrast with two in the parental (SKOV3) cells (Fig. 1, C and D). We had identified previously HNF3α, mapped at 14q12, as a target gene within an amplicon at 14q12-q13 in esophageal squamous cell carcinomas (10). As our present FISH experiments showed four signals of HNF3α in SKOV3/VP cells (Fig. 1d), BCL2L2 appears to reside within more critical region of the amplicon at 14q11-q21 in the VP-16-resistant cell line SKOV3/VP. Moreover, additional FISH experiments showed that the smallest amplicon harboring BCL2L2 lay on the region spanning between markers D14S879 and SHGC-101614 (BACs, RP11–146E13 and RP11–144C18, respectively) in SKOV3/VP cells (Fig. 1f). On Northern blots BCL2L2 showed overexpression consequent on amplification in SKOV3/VP cells, a 3.3-fold increase compared with SKOV3 cells (Fig. 2B).

We examined expression patterns of the six BCL-2 genes mentioned above in 22 of our drug-resistant cell lines, using real-time quantitative reverse transcription-PCR. Results were documented as gene-expression ratios compared between drug-resistant cells and their parent lines (Fig. 3). A 2-fold or greater increase in expression was found for BCL2 in 4 of the 22 resistant lines; for BCL2L1 (BCL-XL) in 1 line; for BCL2L2 in 3; for MCL1 in 1; for BCL2A1 in 1; and for BCL2L10 in 5. This analysis confirmed the overexpression of BCL2L2 in SKOV3/VP cells (Fig. 2B; Fig. 3). MCL1 was up-regulated in HT-29/CTP cells and BCL2L10 in K562/AC cells, in accord with their amplification (Table 2; Fig. 3).

Effect of BCL2L2 on Apoprosis Induced by VP-16. To investigate the effect of BCL2L2 overexpression on induction of apoptosis by an anticancer drug, we treated SKOV3 and SKOV3/VP cells with 100 μg/ml of VP-16 for 24 h and measured apoptosis according to activation of caspase-3. Activity of this enzyme in cell extracts prepared from SKOV3/VP cells was significantly lower than in SKOV3 cells (Fig. 4A).

To examine whether down-regulation of BCL2L2 would sensitize SKOV3/VP cells to VP-16, we pretreated cells with an antisense OPT targeting BCL2L2 mRNA, and then added VP-16. The antisense OPT (AS), but not the control OPT (SC) or the transfecting reagent (Oli-
gofectamine) alone, induced a decrease in BCL2L2 mRNA (Fig. 4B). Down-regulation of BCL2L2 by AS increased the sensitivity of SKOV3/VP cells to Vp-16 compared with pretreatment with SC (Fig. 4C).

**Gene-Copy Numbers and Expression Levels of DCK, TOP1, and TOP2A.** In our CGH analysis, K562/AC cells showed a region of loss at 4q where DCK locates, and HT-29/CPT cells showed a loss at 20q where TOP1 resides, although no changes were seen in 5 VP-
ALTERATION IN GENE-COPY NUMBERS AND DRUG RESISTANCE

Fig. 1. Continued
16-resistant lines at 17q21–22, the location of TOP2A (Table 1). We determined copy numbers of DCK, TOP1, and TOP2A by FISH and quantified their expression levels by real-time reverse transcription-PCR in Ara-C, CPT, and VP-16 resistant cell lines, respectively, to compare the data with expression of those genes in their parental lines. Results are summarized in Table 3.

DCK produced only one FISH signal in K562/AC cells in contrast to two in K562 cells, indicating hemizygous deletion of the gene in the Ara-C-resistant cells (Fig. 1, E and F). Expression of DCK was evident in K562 cells but not detectable in K562/AC cells (data not shown), a feature that had been observed previously (14). HT-29 cells contain five copies of TOP1, but HT-29/CPT cells had only two copies (Fig. 1, G and H). Similarly, TOP1 copy numbers decreased to three in St-4/CPT cells from six in St-4 cells. Expression levels of TOP1 were lower in all 3 of the CPT-resistant cell lines than in their parental lines. TOP2A copy numbers were reduced in HT-29/ETP, K562/etop20, and K562/etop80 cells, and expression of this gene was down-regulated in all 5 of the VP-16-resistant cell lines.

**DISCUSSION**

Proteins of the ABC-transporter family are responsible for the transport of a wide variety of substrates ranging from ions to large proteins (12). Thirteen ABC transporter genes were amplified in our drug-resistant cell lines (Table 2). Among them, ABCB1 (MDR1) and ABCC1 (MRP1) are known to cause resistance to multiple drugs by increasing efflux from cells (15, 16). Forced expression of ABCB11 (BSEP), which was amplified in A549/CPT cells, can induce resistance to Taxol (17). However, to our knowledge no associations with drug resistance have been reported for the other 10 ABC-transporter genes we examined.

Within the amplified region at 16p12-p13 in HT-29/ETP cells, ABCA3 was amplified and overexpressed more prominently than ABCC1, suggesting that the former was a more probable target in the amplicon than the latter. Others have shown that ABCA3 is a lamellar-body membrane protein present in lung alveolar type II cells; it may be involved in the formation of pulmonary surfactant (18). ABCC9 (SUR2), first cloned as an isoform of the sulfonylurea receptor ABCC8 (SUR1), encodes a constituent of ATP-sensitive K+ channels (19). ABCC9 was amplified in SK3/VP16 and SKOV3/VP cells, although our CGH study showed no gain at 12p in SK3/VP16 cells. This discrepancy suggested that the amplicon harboring ABCC9 in SK3/VP16 cells might be too narrow to be detected by CGH, and, therefore, the gene was likely to be specific target. The transcript was in fact up-regulated in SK3/VP16 cells (Fig. 2C).

The ability of BCL-2 to block cell death induced by anticancer...
Fig. 4. A. Caspase-3 activity in SKOV3 and SKOV3/VP cells treated with VP-16. SKOV3 and SKOV3/VP cells were treated with 100 μg/ml of VP-16. VP-16 plus pan-caspase inhibitor Z-VAD-FMK, or medium (control) for 24 h. Cell lysates were incubated with substrate, and absorbance at 405 nm was measured to determine caspase-3 activity. Z-VAD-FMK was added to confirm that the substrate cleavage in fact represented caspase activity. B. BCL2L2 mRNA levels determined by real-time reverse transcription-PCR in SKOV3/VP cells treated with a BCL2L2 antisense oligonucleotide containing phosphorothioate backbones (OPT; AS), a scrambled-sequence control OPT (SC), or the transfecting agent (Oligofectamine) alone. C. sensitization of SKOV3/VP cells to VP-16 by down-regulation of BCL2L2. Cells were treated with AS (○), SC (△), or left untreated (□); 24 h later, VP-16 was added at the indicated concentrations and maintained for 48 h. Cell viability was determined by water-soluble tetrazolium salt assay 72 h after transfection. Percentages were calculated against the absorbance of cells maintained under identical experimental conditions without OPT or VP-16. Each determination was performed in quintuplicate. The values shown on the plots are the means; bars, ± SD.

 drugs has established it as a novel type of multidrug-resistance protein. Several clinical studies support the hypothesis that high-level expression of antiapoptotic BCL-2 proteins such as BCL-2, BCL-XY, or MCL-1 confers a clinically important chemoresistant phenotype on cancer cells (20). All 6 of the BCL-2 genes we examined were up-regulated in several of our drug-resistant cell lines. BCL2L2, MCL1, and BCL2L10 were amplified in SKOV3/VP, HT-29/CPT, and K562/AC cells, respectively, and all were overexpressed in consequence. Our findings indicated that the amplification is capable of activating these antiapoptotic genes.

Enforced expression of BCL2L2 renders hematopoietic cells refractory to several (but not all) cytotoxic conditions (21). BCL2L2-deficient mice undergo more apoptosis in the intestine than wild-type mice after treatment with 5-fluorouracil or γ-radiation (22). In our studies, caspase-3 activity induced by VP-16 was lower in SKOV3/VP cells that showed higher levels of BCL2L2 expression than their parent cells. In addition, down-regulation of BCL2L2 with an antisense OPT sensitized SKOV3/VP cells to VP-16. These findings suggest that BCL2L2 is at least partially responsible for the chemoresistance of SKOV3/VP cells.

Ara-C is converted to the active phosphorylation metabolite 1-β-D-arabinofuranosylcytosine 5′-triphosphate during formation of cytoxic triphosphate derivatives by the coenzyme activity of deoxycytidine kinase (dCK); its cytotoxicity is exerted via incorporation into DNA. Reduced activity of dCK may, therefore, inhibit initial phosphorylation of Ara-C to 1-β-D-arabinofuranosylcytosine 5′-triphosphate, resulting in cellular resistance to Ara-C (14). No expression of dCK has ever been detected in K562/AC cells (14). Our results revealed that DCK was hemizygously deleted in K562/AC cells; the deletion might be attributable to decreased expression of the transcript, although additional unknown mechanism(s) must be involved in inactivation of the residual allele.

Topoisomerase (Topo) I and Topo II α cause breaks in single- and double-stranded DNA, allowing strands to separate during replication. CPT and VP-16 inhibit Topo I and Topo II α, respectively, after breakage has occurred, leading to the persistence of broken strands and, therefore, to inhibition of replication. Increases in these two enzymes lead to increased drug sensitivity, whereas decreases lead to resistance (1). We demonstrated that forced expression of TOP1 induced higher sensitivity to CPT (23). McLeod and Keith (24) showed that copy numbers of the TOP1 gene were correlated with TOP1 expression and inversely correlated with sensitivity to SN38, a derivative of CPT, in a panel of breast and colon cancer cell lines. Withoff et al. (25) reported fewer copies of TOP2A in a panel of doxorubicin-resistant cell lines.

Our earlier study had revealed that cellular contents of Topo I were decreased in CPT-resistant cell lines (26), although the mechanism remains unknown. In the present study, expression levels of TOP1 were lower in all 3 of our CPT-resistant cell lines, and HT-29/CPT and St-4/CPT cells contained fewer copies than their parent cells. Amplification of 20q, where TOP1 is located, is frequent in colon and gastric cancers (27); indeed, in our study TOP1 copy numbers were sometimes increased, to five in HT-29, and to six in St-4. However, in the process of acquiring resistance to CPT several copies of the gene had been lost in HT-29/CPT and St-4/CPT cells. Yanase et al. (23) detected expression of two species of TOP1 mRNA in HT-29/CPT; one was wild-type and another was a deleted mutant. Combined with our observations, that information suggests that one of the two copies of TOP1 in HT-29/CPT expresses the deleted mutant and thereby contributes to additional reduction of TOP1 expression.

Expression levels of TOP2A were lower in 5 VP-16-resistant cell lines than in their parental lines, and copy numbers of this gene decreased in 3 of those 5 lines. Collectively, our results suggest that the decrease in copy numbers of TOP1 and TOP2A resulted in the reduction of their expression in CTP- and VP-16-resistant cell lines, respectively, and led to acquired resistance to those drugs.
We have shown here that alteration of gene-copy number is one of the mechanisms capable of activating or down-regulating genes that are implicated in acquired chemoresistance. Therefore, CGH analyses may yield new hallmarks for identifying novel genes involved in that process. Clinical samples from patients with intrinsic or acquired resistance to chemotherapeutic agents should be investigated now, to identify additional target genes within the genomic regions where we have identified changes characteristic of drug-resistant cells, and to clarify the functions of those genes.

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