Localization of a Novel Multidrug Resistance-associated Gene in the HT1080/DR4 and H69AR Human Tumor Cell Lines

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Abstract

Two doxorubicin-selected human tumor cell lines, H69AR and HT1080/DR4, display a multidrug resistance phenotype but do not overexpress P-glycoprotein. Recently, a 6.5-kilobase mRNA encoding a novel member of the ATP-binding cassette superfamily of transport proteins, designated multidrug resistance-associated protein (MRP), has been identified in the H69AR cell line. In the present study, the levels of MRP mRNA were found to be 14-fold higher in HT1080/DR4 cells relative to sensitive HT1080 cells. Southern blotting indicates that gene amplification contributes to the overexpression of MRP in HT1080/DR4 cells. Using a 4-kilobase MRP complementary DNA probe, MRP genes were localized to 2-5 chromosomes bearing homogeneously staining regions and to multiple double minute chromosomes in H69AR cells. Resistant H69AR cells also contained a new der(16) with a structural aberration affecting 16p13.1, the normal cellular locus of the MRP gene. The MRP probe hybridized to two small homogeneously staining regions (hsr) in HT1080/DR4 cells including hsr(7)(p12p15). MRP localization was restricted to the normal cellular locus, 16p13.1, in the parental H69 and HT1090 cells and the drug-sensitive H69PR revertant cells. Our data provide combined evidence that amplification of the MRP gene is associated with expression of drug resistance in selected solid tumor cell lines.

Introduction

Multidrug resistance is a complex and multifactorial phenomenon that limits the efficacy of cancer chemotherapy. Overexpression of a Mr 170,000 plasma membrane protein, P-gp, is found frequently in many drug-resistant cell lines (1). However, it is apparent that reduced drug accumulation mediated by P-gp constitutes only one of several biological mechanisms involved in the development of a multidrug-resistant phenotype. HT1080/DR4 is a DOX-selected multidrug-resistant human fibrosarcoma cell line that does not overexpress P-gp (2, 3) and the mechanisms responsible for resistance in this cell line remain unclear (4). The multidrug-resistant small cell lung carcinoma cell line, H69AR, also does not overexpress P-gp (5, 6) but recently, a cDNA encoding a novel MRP has been cloned from this cell line (7). MRP belongs to the superfamily of ATP-binding cassette transport systems. Other members of this superfamily include the cystic fibrosis transmembrane conductance regulator, P-glycoprotein, and the MHC class II-linked peptide transporters (7, 8). The 6.5-kilobase MRP mRNA is increased approximately 100-fold in H69AR cells relative to drug-sensitive parental H69 and revertant H69PR cells. This increase is associated with a 40-50-fold amplification of the MRP gene which maps in normal cells to chromosome band, 16p13.1 (7). In the present study, we have found that the MRP gene is also amplified in resistant HT1080/DR4 cells relative to parental HT1080 cells. To identify the cytogenetic alterations associated with amplification of the MRP gene, we have carried out cytogenetic and FISH analyses on the sensitive and resistant HT1080 cell lines and the sensitive, resistant, and revertant H69 cell lines.

Materials and Methods

Cell Lines. The two DOX-selected multidrug-resistant cell lines used in this study have been described previously (2, 5, 9). The 200-fold DOX-resistant HT1080/DR4 fibrosarcoma cell line was derived from parental HT1080 cells and exhibits a multidrug-resistant phenotype (2, 9). H69AR is a 50-fold DOX-resistant small cell lung carcinoma cell line that also displays a typical multidrug-resistant phenotype (5, 10). H69PR, a drug-sensitive revertant cell line, was derived from H69AR by maintaining H69AR cells in DOX-free medium for 36 months (11). The resistant HT1080/DR4 and H69AR cell lines do not overexpress P-gp (2-6).

RNA and DNA Analyses of HT1080 Fibrosarcoma Cell Lines. Polyadenylated RNA was obtained with a Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA) and 1 μg from each cell line was separated by electrophoresis on a formaldehyde-agarose denaturing gel. The RNA was transferred to a nylon membrane (Zetaprobe; Bio-Rad, Mississauga, Ontario, Canada), prehybridized in 50% formamide, 5X SSC-5X Denhardt's (50X = 1% bovine serum albumin, 1% polyvinylpyrrolidone, and 1% Ficoll), 1% SDS, and sheared and denatured herring testes DNA (100 μg/ml) for 4.5 h at 42°C. The blot was hybridized with a 1.8-kilobase DNA fragment of MRP labeled to a specific activity of >5 x 106 cpm/μg DNA with [α-32P]dCTP (3000 Ci/mmol; NEN-Dupont, Mississauga, Ontario, Canada) by random priming (12). Hybridization was carried out for 16-20 h at 42°C. Blots were washed once in 2X SSC for 10 min at room temperature and then three times in 0.1% SDS and 0.1X SSC for 30 min each at 52°C followed by autoradiography. To estimate variation in RNA loading of the gel, the blots were stripped and reprobed with a 32P-labeled cDNA for β-actin (201pbv2.2) (13). Relative levels of MRP and β-actin mRNAs were determined by densitometry (7).

For Southern analyses, genomic DNA (10 μg) from HT1080 and HT1080/DR4 cells was digested with EcoRI and BamHI and electrophoresed through a 0.8% agarose gel. After blotting onto a nylon membrane (Zetaprobe), prehybridization was carried out for 4 h at 42°C in 50% formamide, 5X standard saline-phosphate-EDTA (1X = 3 μM NaCl-0.2 μM Na2HPO4, 0.02 M EDTA, pH 7.4), 0.5% SDS, 4X Denhardt's, and herring testes DNA (100 μg/ml). The blot was then hybridized for 20 h at 42°C with a 4-kilobase MRP cDNA fragment labeled by random priming with [α-32P]dCTP as described above. The blot was washed once in 1X SSC for 20 min at room temperature, and twice with 0.1X SSC and 0.1% SDS for 20 min at 42°C and then exposed to film. Densitometry was carried out as before.

Classical Cytogenetics. Cytogenetic analyses were performed according to the standard method using 50 μg/ml Colcemid for 45 min before cell harvesting. GTG-banding was used to identify the individual chromosomes and the chromosomes were classified according to the Guidelines for Cancer Cytogenetics, Supplement to An International System for Human Cytogenetic Nomenclature (14).
Fluorescent in Situ Hybridization. FISH studies using a biotin-labeled chromosome 16-specific paint (Oncor, Gaithersburg, MD) and a 4-kilobase fragment of the MRP cDNA were performed following GTG-banded cytogenetic studies. The MRP cDNA probe was biotin-labeled by nick translation (Oncor nonisotopic probe labeling kit). Slides containing metaphase spreads were treated per manufacturer's instructions for the Oncor chromosome in situ system for single copy sequence detection in metaphase chromosomes using fluorescein isothiocyanate-labeled avidin-anti-avidin antibody for amplification of the signal. Metaphase analysis and photomicroscopy were performed using a Nikon photomicroscope. Photographs were taken using either Kodak Ektachrome 100 color slide film or the ACLAIMS imaging system (W-Technology, Sacramento, CA).

Results and Discussion

The two DOX-selected multidrug-resistant cell lines, HT1080/DR4 and H69AR, display drug resistance patterns typical of that associated with increased amounts of P-gp, the M, 170,000 plasma membrane glycoprotein that is presumed to confer resistance by reducing cellular drug accumulation (15). However, overexpression of P-gp is not found in either cell line (2–6). Furthermore, DOX resistance in these two cell lines is poorly reversed by various chemosensitizing agents that are known to reverse P-gp-mediated multidrug resistance (16). Increased levels of intracellular drug detoxification enzymes (i.e., glutathione S-transferase, glutathione peroxidase) or quantitative or qualitative alterations in drug targets such as topoisomerase II, do not appear responsible for the relatively high levels of drug resistance observed in these two model systems nor can they account for the cross-resistance of these cell lines to the Vinca alkaloids (4, 6, 10). These data suggested that alternate mechanisms of multidrug resistance are responsible for acquired drug resistance in these cell lines.

Initial studies were attempted to determine if amplified genes contributed to the HT1080/DR4 non-P-gp-mediated multidrug resistance phenotype using the in-gel renaturation technique; however, this method could not detect amplified genes less than 25-fold increased (3). Recently, a 6.5-kilobase MRP mRNA was isolated from the non-P-gp-mediated multidrug-resistant H69AR cell line (7). In the present study, we found that MRP mRNA is elevated about 14-fold in the multidrug-resistant HT1080/DR4 fibrosarcoma cells compared to the sensitive HT1080 cells (Fig. 1A). The MRP gene copy number in HT1080/DR4 cells appears to be approximately 5-fold greater than that in the HT1080 cells (Fig. 1B). This suggests that gene amplification contributes to the elevation of MRP mRNA in HT1080/DR4 cells and the resistant, sensitive, and revertant small cell lung cancer cell lines (H69, H69AR, and H69PR).

H69 parental cells were characterized by a near-tetraploid modal chromosome number containing several of the chromosomal aberrations previously described in this cell line by Whang-Peng et al. (17) including a del(11)(q23), der(19)(t;19), del(17)(p12), and dmins. The frequency of dmins ranged from 3 to 105/cell (Fig. 2A). These dmins have been shown previously to house amplified N-myc genes (18). No hsr-bearing chromosomes were identified in H69 parental cells. The MRP gene has been mapped previously by radioisotopic chromosomal in situ hybridization to band 16p13.1 in normal peripheral blood mononuclear cells (7). In H69 cells, two morphologically normal chromosome 16s and two derivative chromosome 16s [der(16)] resulting from unbalanced 5;16 translocations (short and long forms) were identified by GTG-banded analysis (Fig. 2A). To confirm the status of the chromosome 16 markers and to rule out dmins bearing chromosome 16 material, FISH analysis using a chromosome 16 paint was performed. Four bright signals indicative of the two normal chromosome 16s and two der(16)s were identified (Fig. 2B). The chromosome 16 paint did not hybridize with the dmins in the parental cells (Fig. 2B), a result agreeable with the previously reported N-myc amplified gene sequences derived from chromosome band, 2p24.1 (18).

The karyotype of the resistant H69AR cell line revealed substantial karyotypic changes from its parental H69 cell line. The chromosome mode of H69AR was near-triploid with multiple numerical and structural changes. Of interest, only one morphologically normal chromosome 16 and one der(16) present in the H69 cells were observed in H69AR cells, although a new der(16) with a structural alteration involving band 16p13.1 appeared in the resistant cells. The mainline karyotype of H69AR contained two large hsr-bearing marker chromosomes (Fig. 3), whereas various smaller hsr localized to chromosome 1, 6, and 7 [der(1), der(6), and der(7)] in other H69AR cells (Fig. 3, Inset A). The number of dmins was similar to the parental cells with a range of 2–100/cell (Fig. 3, Inset B). The chromosome 16 paint hybridized to the normal chromosome 16, the hsr-bearing chromosomes, and a population of dmins (Figs. 2C and 3, bracketed chromosomal regions). These data demonstrate the presence of chromosome 16 material in both hsr and dmins in the same cell. Karyotypic evaluation of the revertant small cell lung cancer cell line, H69PR, revealed four der(16)s including one morphologically normal chromosome 16, one to two copies of the der(16) [der(16)(5;16)], and another der(16) with a putative hsr-bearing region on the short arm which may have been derived from the der(16) chromosome with a structural aberration involving band 16p13.1 in H69AR cells (Fig. 2D). Dmins were found in the revertant H69PR cells but, as in the H69 and H69AR cell lines, the number per cell was highly variable ranging from 0 to 142 dmins/cell. The chromosome 16 paint showed that the putative hsr on 16p was not composed of chromosome 16 material (Fig. 2E). In addition, no dmins hybridized with the 16 paint implying that only the N-myc amplified dmin population is present in H69PR cells as was the case in H69 cells. These data indicate that the cytogenetic markers of gene amplification, namely, the hsr-bearing...
Fig. 2. Cytogenetic and FISH studies of the drug-sensitive and multidrug-resistant cell lines. A, GTG-banded metaphase of H69 cells. Arrows, two morphologically normal chromosome 16s. Arrowheads, two der(16)(5;16). Note multiple dmins. B, same GTG-banded H69 metaphase destained and hybridized with a chromosome 16 paint. The chromosome 16s and der(16)s hybridize with the chromosome 16 paint; however, the dmins do not hybridize. C, chromosome 16 painting of H69AR cells. Multiple chromosomes and many of the dmins hybridize with the chromosome 16 paint. D, GTG-banded metaphase of H69PR cells. Arrows, the der(16)(5;16) [long form] and the normal chromosome 16. Arrowhead, the new putative hsr-bearing derivative chromosome 16. E, FISH of the same GTG-banded metaphase of H69PR. Note the short arm of the putative hsr-bearing chromosome 16 does not hybridize with the chromosome 16 paint indicating that this material is derived from another chromosome(s). F, MRP hybridization in H69AR cells. Note that the probe hybridizes with the hsr and dmins described in C and Fig. 3. G, MRP hybridization in H69 cells. Hybridization is observed at the normal cellular locus only, 16p13.1 (arrow) of the der(16)(5;16), short form. This chromosome has been enhanced to visualize the minute signal using the ACLAIMS Image Analysis System. H, GTG-banded metaphase of HT1080 parental cells. Arrows, four chromosome 16s. I, chromosome 16 painting of the same HT1080 metaphase identifies four chromosome 16s. (The signal of one chromosome 16 is faint due to overlapping chromosomes.) No cryptic translocations involving chromosome 16 were identified. J, HT1080/DR4 GTG-banded metaphase. Arrows, hsr(7)(p12p15) and the small hsr marker chromosome. K, same HT1080/DR4 cell after FISH using a chromosome 16 paint. Ten sites contain chromosome 16 material in HT1080/DR4 cells. The striped appearance of hsr(7)(p12p15) indicates that material from other chromosomes is present in this region. L, MRP hybridization in HT1080/DR4 cells. Green arrows, small hsr housing amplified MRP genes in HT1080/DR4 cells which correspond to the GTG-banded chromosomes in 2J (arrows). White arrows, normal MRP cellular locus, 16p13.1. These signals were enhanced using the ACLAIMS image analysis system. Note the different MRP hybridization patterns between H69AR (F) and HT1080/DR4 (L).
chromosomal markers and a population of dmins containing chromosome 16 material observed in the H69AR cells, are no longer present in the revertant H69PR cell line.

Using a 4-kilobase MRP cDNA probe, FISH analyses confirmed that the hsr-bearing chromosomal markers as well as a population of dmins in resistant H69AR cells that hybridize with the chromosome 16 paint contained amplified MRP genes (Fig. 2F). The MRP cDNA hybridized only to chromosome band 16p13.1 in the sensitive parental (Fig. 2G) and revertant (data not shown) cell lines, thus confirming the localization of the MRP gene to its native site. Interestingly, the new der(16) with a structural aberration affecting the short arm of chromosome 16 at band 16p13.1 was observed in the resistant cells. Carroll et al. (19) provide evidence that an early step in gene amplification is a recombination event which deletes a chromosomal region containing a replication origin along with adjacent genes. Alternatively, an intrachromosomal rearrangement with a subsequent translocation may give rise to bridge/breakage/fusion amplification cycles (20). The chromosomal rearrangement involving the short arm of chromosome 16 in H69AR would support either of these two possibilities. Increases in gene copy number could occur rapidly as the acentric elements partition unequally at mitosis leading to the selective overgrowth of the MRP dmins-bearing cells in the presence of DOX. Subsequent selection appears to induce integration of dmins and the formation of hsr.

Chromosomal markers were also evaluated in the HT1080 fibrosarcoma cells. The parental HT1080 cells contained a chromosomal mode of near-tetraploid with two copies of the chromosomal markers [add (5)(p15) and t(3;11)] described previously for this cell line (9). Four morphologically normal chromosome 16s were identified in HT1080 parental cells using a chromosome 16 paint (Fig. 2H and I). HT1080/DR4 cells were characterized by a hypotriploid modal range and a strikingly complex karyotype. FISH studies using a chromosome 16 paint revealed eight to ten signals representing multiple rearrangements involving chromosome 16 in the HT1080/DR4 cells (Fig. 2K). These included a previously identified hsr-bearing chromosome 7 (9) and a smaller unidentified marker chromosome. The striped hybridization pattern of the hsr(7p) with the chromosome 16 paint indicates that material from chromosomes other than chromosome 16 is present in this region. Consequently, these molecular cytogenetic findings indicate that this chromosomal region would be more accurately defined as an abnormally banding region or abr [a descriptive term to describe a banding pattern that is not accepted as conventional nomenclature by ISCN (14)]. The present results are in agreement with the complex karyology described for HT1080/DR4 during its selection in DOX (9). However, when FISH was carried out with the MRP cDNA probe, hybridization was observed only in the hsr inserted in the short arm of chromosome 7, a small marker chromosome, and the normal cellular locus, 16p13.1 (Fig. 2, J and L). Taken together, the complex karyotypic changes observed involving chromosome 16 in H69AR resulting in multiple hsr and dmins housing amplified MRP gene sequences (Fig. 2C) are strikingly different when compared to HT1080/DR4 cells (Fig. 2K). These data suggest that additional factors might be involved in the multidrug resistance phenotype of HT1080/DR4.

In conclusion, the present study provides evidence that overexpression of MRP is associated with multidrug resistance in the HT1080/DR4 cell line. Our cytogenetic data are consistent with the DNA analyses indicating a gene amplification mechanism contributes to overexpression of MRP. However, it will not account completely for the relative increase in MRP mRNA. While the level of MRP mRNA in H69AR cells relative to H69 cells is roughly parallel to the level of DOX resistance, this is not the case with HT1080/DR4 cells. The latter cell line is 200-fold resistant to DOX but MRP mRNA is elevated only 14-fold compared to drug-sensitive HT1080 cells. However, we do not know at present how the levels of MRP protein compare in the two resistant cell lines. These observations suggest that
while MRP may play a role in HT1080/DR4 resistance, it remains possible that other mechanisms contribute as well. It is also worth noting that although both the cytogenetic and DNA analyses indicate that MRP is amplified in the two cell lines, the cytogenetic alterations associated with MRP gene amplification are quite dissimilar. H69AR cells were characterized by 2–5 large hsrS with multiple dmins (Fig. 2F) housing approximately a 50-fold increase of MRP gene sequences whereas the 200-fold HT1080/DR4 cells contained only two small regions (Fig. 2L) containing a 5-fold increase in MRP gene sequences. Collectively, these data suggest that the MRP gene is overexpressed in multidrug-resistant cell lines derived from solid tumors. Studies are in progress to determine the molecular basis of the acquired multidrug resistance in HT1080/DR4. Further studies are necessary to determine the role of MRP and other possible factors in clinical drug resistance.

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