Short Communication

Genomic Imbalances Associated with Acquired Resistance to Platinum Analogues

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During the past several years, a panel of human tumor cell lines (predominantly ovarian) with acquired resistance to cisplatin, the orally bioavailable analogue JM216, and the structurally hindered analogue AMD473, has been established and characterized for underlying mechanisms of resistance. We have examined these resistant cell lines for gains and losses of DNA associated with the acquisition of resistance using the molecular cytogenetic technique of comparative genomic hybridization. Our comparison of three analogues has shown the most frequently observed changes to include amplification of 4q (5/7) and 6q (5/7), followed by amplification of 5q (3/7). We have defined four minimal common overrepresented regions, two each on 4q and 6q, which are potential loci of genes associated with platinum analogue resistance. Additional consistent abnormalities appear to be associated with cell lines sharing specific resistance mechanisms. For example, amplification of 12q was observed in the CH1 lines made respectively resistant to JM216 and AMD473 in which increased DNA repair appears to be a major mechanism of resistance for both agents. Hence, these comparative genomic hybridization studies have identified distinct chromosomal aberrations which may correlate with defined mechanisms of resistance and contain hitherto unrecognized genes that may provide targets for future therapeutic intervention. (Am J Pathol 1999, 155:77–84)

The major thrust in anticancer therapeutic development is the identification of selective therapies against molecular targets.[1,2] The identification of molecular mechanisms of drug resistance has been expedited by the examination of cell lines with acquired resistance, using modern molecular techniques. These techniques include classical cytogenetics, differential display, fluorescent *in situ* hybridization (FISH), and the more modern approaches of comparative genomic hybridization (CGH) and spectral karyotyping (SKY).

CGH is a new technique used to examine an entire genome for variations in DNA sequence copy number[3] (amplifications and deletions). It does not require replicating cells and therefore produces results which are representative of the tumor as a whole and not just the dividing population. In contrast to FISH, it does not require a previous knowledge of genetic aberrations. It can be employed with DNA extracted from fresh tumor material or material that has been frozen, formalin-fixed, or paraffin embedded. Finally, in contrast to differential display, CGH provides information on the chromosomal location of the amplified or deleted region. We have used DNA from corresponding pairs of resistant and sensitive cell lines labeled with fluorochromes of different colors, eg, green and red. These two DNAs are hybridized simultaneously to metaphase spreads from control (normal) cells. Comparison of the ratio of red:green signal along each chromosome axis reveals regions of gain and loss between the sensitive and resistant cell lines.

CGH is currently being used to determine aberrations in solid tumors and hematological malignancies in order to identify changes common to particular sub-types of tumor.[4,5] For example, high-level amplifications of regions of chromosomes 17q and 20q have been identified in breast carcinoma.[6] In a few instances, these findings have correlated with prognostic significance, eg, the identification of amplification of the REL proto-oncogene in diffuse large cell lymphoma has been associated with progression of disease.[7]

We have confirmed that genetic loci associated with known mechanisms of resistance show the corresponding chromosomal imbalance with CGH; for example, we
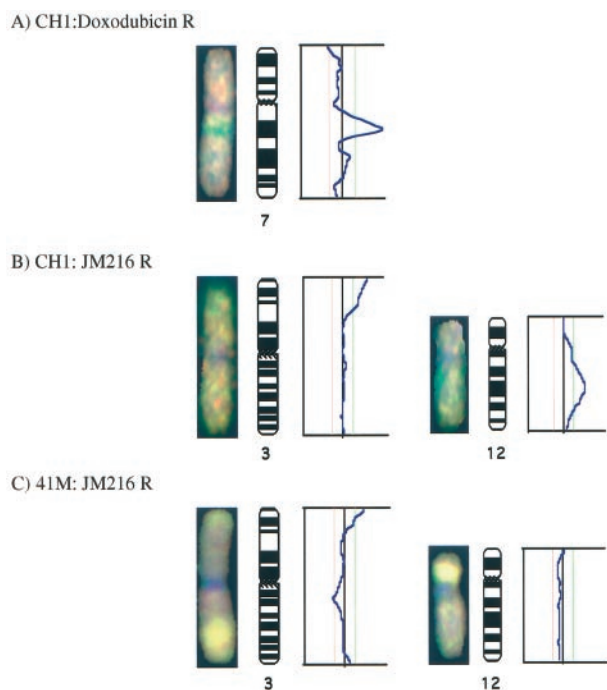
Figure 1. Individual chromosomes and their corresponding mean ratio profiles are shown for chromosomes with significant imbalances associated with acquired resistance to Doxorubicin in the CH1 cell line (**A**), JM216 in the CH1 cell line (**B**), and JM216 in the 41M cell line (**C**). The average of the red:green ratios along the axis for a number of copies of each chromosome is indicated by the blue line in each graph. The red and green dotted lines indicate the significance level (confidence limits of 99%) for 30% of the cells having the imbalance in a diploid population.

have demonstrated that the CH1 cell line with acquired resistance to doxorubicin shows significant amplification of the P-glycoprotein (MDR1) gene (Figure 1). Other authors have also shown amplification of this region of 7q21 in cell lines with classical multi-drug resistance using CGH[8] and reverse *in situ* hybridization.[9] Moreover, in a cell line with acquired resistance to etoposide known to be mediated through topoisomerase II, we have demonstrated deletions of both the topoIIα gene on chromosome 17q21–22 and the topoIIβ gene on chromosome 3p24–25.

A panel of acquired cisplatin-resistant human tumor cell lines (predominantly ovarian) has previously been established and characterized for the underlying mechanisms of resistance to cisplatin in order to support our mechanism-directed approach to the circumvention of resistance.[10,11] Previous studies have indicated that cisplatin is able to circumvent acquired resistance due to reduced drug transport (eg, in the 41M:cisR line[12]). Orr et al have also reported recently that JM216 (and other ammine/amine platinum (IV) dicarboxylates) are able to circumvent acquired resistance to cisplatin, carboplatin, and tetraplatin in murine L1210 leukemia sublines.[13] A study has been undertaken to determine whether cisplatin resistance mechanisms are also involved in acquired resistance to JM216 using two cell lines (41M and CH1) with derived resistance to JM216. Interestingly, and in contrast to its acquired cisplatin-resistant counterpart, JM216 resistance in the 41M cell line appeared to be due

to enhanced glutathione levels, rather than drug transport.[14] In addition, following 2 years of exposure to JM216, only a relatively low level of resistance (1.9-fold) was achievable. These data suggest that acquired resistance to JM216 is less likely to occur through reduced drug accumulation, a common mechanism of resistance in acquired cisplatin-resistant cell lines. In the CH1: JM216-resistant cell line, in common with its cisplatin-resistant counterpart, acquired resistance appeared to be due to the increased DNA repair of JM216-induced adducts. Half-times for the removal of total platinum bound to DNA after JM216 exposure were 20 hours for the parental CH1 line, in contrast to 11 hours for the CH1:JM216R line. The broad-spectrum third-generation platinum drug AMD473 was designed to circumvent tumor resistance to cisplatin and carboplatin by being less susceptible than cisplatin to thiol binding. AMD473 was evaluated against our established panel of human ovarian carcinoma cell lines containing examples of acquired cisplatin resistance mediated through defined mechanisms.[10–12] AMD473 has shown promising circumvention of acquired cisplatin resistance in many of these *in vitro* human ovarian cell lines, which included specific models of acquired cisplatin resistance.[15]

We report here for the first time the examination of these resistant cell line models for genomic aberrations associated with the acquisition of resistance using the molecular cytogenetic CGH technique. The profiles of three different platinum analogues (cisplatin, JM216, AMD473) have been compared in the same cell line, and the same members of the platinum family have also been studied in three different cell lines (CH1, 41M, and A2780).

Materials and Methods

Cell Lines

Ovarian carcinoma cell lines 41M and A2780 were established from previously untreated patients. CH1 was established from an ovarian carcinoma patient previously treated with and resistant to cisplatin and carboplatin. The resistant cell lines were established by serial passage in the presence of increasing concentrations of drug.[16,17] The resistance was stable for at least 6 months and did not require any maintenance dosing with cisplatin or JM216. All cell lines were maintained as monolayers in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum, 50 μg/ml gentamicin, 0.5 μg/ml hydrocortisone, and 2 mmol/L L-glutamine in a 10% CO$_2$ atmosphere.

Drugs

Cisplatin

Cis-diamminedichloroplatinum(II) was first shown to have antitumor activity in 1969[18] and has subsequently become a pivotal component of many therapeutic regimens against a wide variety of solid tumors. However, its clinical use is severely limited by both nephrotoxicity and neurotoxicity. Hence, numerous attempts have been

Table 1. Resistance Levels and Chromosome Aberrations Associated with Acquired Resistance to Platinum Analogues

Cell line: Selection agent	Resistance	Sites of chromosomal imbalance
CH1: cisplatin R.	6.4-fold	+Xq24–28, +4q13–27, +7.
41M: cisplatin R.	4.7-fold	−3q24–29, +4q23–28, −5p12–15.3, +5q11.2–31, +6q16–27, +7p11.2–22, −7q32–36, −9p21, +10q11.2–24, −12q22–24.3.
A2780: cisplatin R.	16-fold	+1q21–23, −1q32–44, +6q21–27, −13q14–34, +17q22–25.
CH1: JM216 R.	6.2-fold	+3p21–26, +12q13–24.1, −18q21–23.
41M: JM216 R.	1.9-fold	+3p23–26, −3q13.2–13.3, +4q21–23, +5p13–q23, +6p12–q12, +6q25–27, −8q13, −9p12–21, +9q22–32, +14q12–13, −14q24–32, +17q21–25.
CHI: AMD473 R.	3- to 4-fold	−Xp21–22.3, +2p14, +4q26–33, +5p14, +6q15–23, +9p21–24, +11q22, +12q13–23, −17p11–13, +20p11.2–q13.3.
A2780: AMD473 R.	3- to 4-fold	−Xp21–22.1, +Xq21, +4q13–31.3, +5q21–23, +6, −7q11.2–21, −10p11.2–13, −12q23–24.1, −17p11–13, +18p11.1–11.3, −20.

made to synthesize derivatives with an improved therapeutic index.

has activity in several cisplatin-resistant human ovarian cancer cell lines *in vitro*.
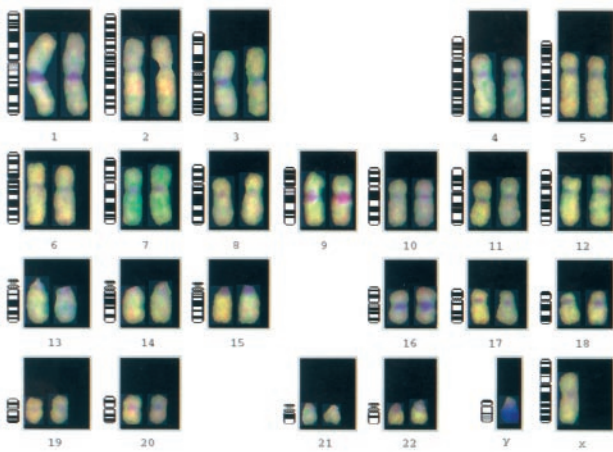
JM216

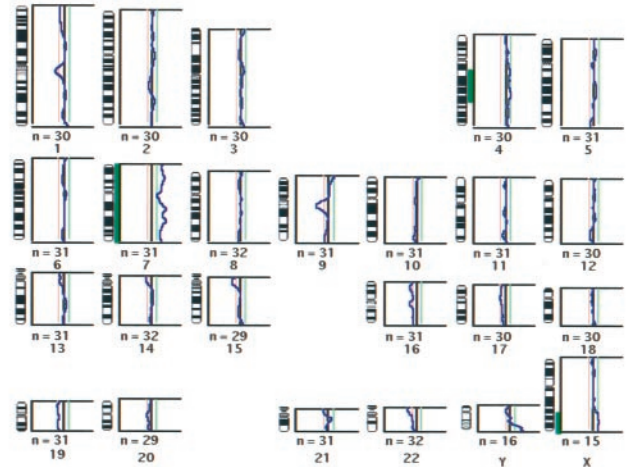Bis-acetato-amminedichloro(cyclohexylamine)platinum(IV) was developed to be orally bioavailable. It

AMD473

Cis-amminedichloro-(2-methylpyridine)-platinum(II) was designed to introduce steric hindrance close to the platinum
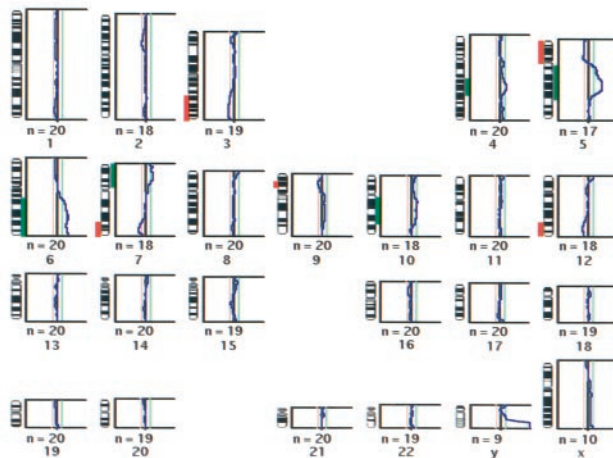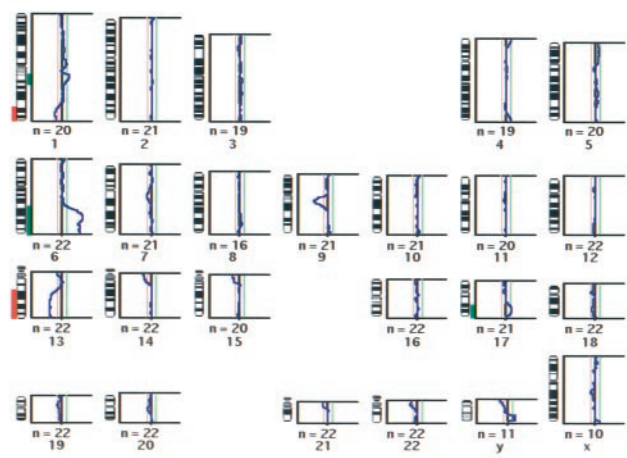

Figure 2. CGH karyotype for the cell line CH1 (**A**) and mean ratio profiles, associated with acquired resistance to cisplatin, are shown for the cell lines CH1 (**B**), 41M (**C**), and A2780 (**D**). Visual examination of the karyotype (**A**) confirms that both the sensitive and resistant DNAs have labeled and hybridized successfully, and also provides an indication of areas of imbalance; the amplification of chromosome 7 is clearly significant on this examination. The areas of imbalance are indicated next to the chromosome ideograms, red for deletion and green for amplification, so that the chromosome location can be identified. For the cell line CH1 (**B**), significant amplifications of 4q, 7, and Xq are apparent. For the 41M cell line (**C**), significant amplifications of 4q, 5q, 6q, 7p, and 10q and deletions of 3q, 5p, 7q, 9p, and 12q are demonstrated. For the cell line A2780 (**D**), significant amplifications of 1q, 6q, and 17q and deletions of 1q and 13q are shown.

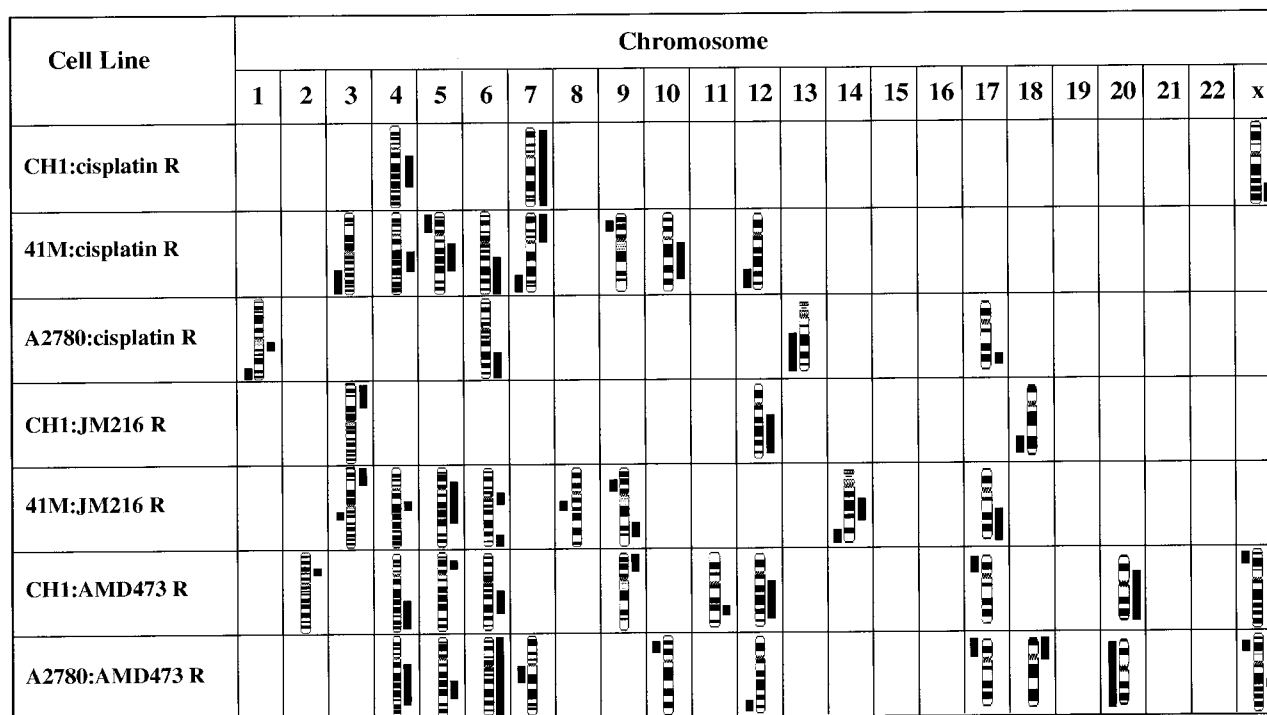| Cell Line	Chromosome																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	x
CH1:cisplatin R																							
41M:cisplatin R																							
A2780:cisplatin R																							
CH1:JM216 R																							
41M:JM216 R																							
CH1:AMD473 R																							
A2780:AMD473 R																							

Figure 3. Amplifications (right) and deletions (left) of genomic sequences associated with acquired drug resistance to platinum analogues.

center through the 2-methylpyridine ligand, thereby reducing the drug's proclivity for deactivation by endogenous thiol-containing species.

CGH

One microgram of DNA from each sensitive and resistant cell line pair was labeled with digoxigenin or biotin respectively, using High-Prime (Boehringer Mannheim, Lewes, UK). Unincorporated nucleotides were removed using Nick columns (Pharmacia, St. Albans, UK). The labeled DNAs were ethanol precipitated together with a 50-fold excess of COT1 DNA (Gibco/BRL, Paisley, UK), resuspended in 10 μl Hybrisol VIII (Oncor, Gaithersburg, MD) and denatured at 75°C for 8 minutes. This mixture was then used to probe slides containing normal male lymphocyte metaphases (Vysis, Downers Grove, IL) as previously described.[19] Following stringent washing, the hybridized signals were detected using fluorescin-avidin and rhodamine-antidigoxigenin, and counterstained with DAPI, again under conditions previously described.[19]

Computer-Assisted Image Analysis

Slides were examined using an Axioskop microscope (Carl Zeiss, Welwyn Garden City, UK) equipped with appropriate filters. Separate images were collected for red, green, and blue fluorescence, using a CCD camera (Photometrics, Tuscon, AZ) and Quips software (Vysis). At least 10 metaphases were captured for each hybridization, the chromosomes were karyotyped, and the axis defined based on the DAPI banding pattern. The DAPI image was used as a mask for the red and green images

to exclude background fluorescence. The total image intensity for the masked red and green images was then independently normalized to accommodate differences in the image capture, so that the average red:green ratio for each cell was 1.0. The red:green ratios for sections of each chromosome were measured perpendicular to the axis. Chromosomal imbalances affecting more than 30% of the cell population, were identified where the ratio was greater than 1.15–1.20 for gains and less than 0.85–0.80 for losses (all imbalances were within 99% confidence limits). Chromosomes 1p, 16, 19, and 22 have previously been reported as having an unreliable CGH profile due to a variable number of interspersed repetitive elements between individuals.[20] We have therefore considered only imbalances affecting more than 50% of the cells in these regions to be significant.

Results

Regions of chromosomal imbalance associated with acquired resistance were seen in all the cell lines examined, varying in number from 3 to 10. The specific chromosome band locations of all the imbalances observed are listed in Table 1. All the amplifications observed were low level (less than 5 extra copies). This contrasts with the high level amplification of the MDR1 gene observed in the CH1:doxorubicin resistant cell line (see Figure 1A): however, this CH1:doxorubicin cell line is approximately 80-fold resistant to doxorubicin whereas the highest level of resistance of any of the platinum analogue resistant cell lines in this study is 11-fold.

Table 2.   Chromosome Localization of Genes Implicated in Platinum Resistance

	Chromosome location
Genes involved in signal transduction pathways	
EGF[28] (epidermal growth factor)	4q25
CyclinB p34[29]	5q12
PKCθ[30,31] (protein kinase C)	10p15
CALM1[32] (calmodulin)	14q24–31
PKCβ[30,31]	16p11
HER2/NEU[33]	17q21.2
PKCα[30,31]	17q22–23
Genes involved in the recognition of DNA damage and repair	
TopoIIβ[26,34]	3p24–25
HMG2[35]	4q31
POLB[36,37] (DNA polymerase β)	8p11.2
DDB1[38] (DNA damage binding protein) (xeroderma pigmentosum E)	11q12–13
HMG1[39] (high mobility group protein 1)	13q12
TopoIIα[26,34]	17q21–22
ERCC1[40] (excision repair complementing defective in chinese hamster)	19q13
POLA[37] (DNA polymerase α)	Xp21–22
HSSB[41] (human single-stranded DNA binding protein)	n/a
SSRP1[42] (structure specific recognition protein)	n/a
Other genes implicated in modulating cisplatin resistance	
JUN[30]	1p31–32
MLH1[43]	3p21–23
WAF1[44] (wildtype p53 activated fragment)	6p21
PMS2[43] (post meiotic segregation)	7p22
MDR1[26] (multidrug resistance)	7q21
MYC[45]	8q24
H-RAS[46]	11p15
GSTπ[47]	11q13
MDM2[48]	12q14–15
FOS[49]	14q24
MRP[26,34] (multidrug resistance protein)	16p13
LRP[34] (lung resistance protein)	16p11
Metallothioneins[50]	16q13
p53[44,48]	17p13
ERBB2[51]	17q21
BCL2[52]	18q21.3
PLS3[53] (T-plastin)	n/a
HSP-60[54] (heat shock protein)	n/a
BCLX[55]	n/a

It is clearly critically important to dissociate genes associated with drug resistance from those associated with growth advantage. We have currently reported the extreme variability developed in four different sub-cultures of the MCF7 breast cancer cell line.[21] Hence, we were scrupulous in every case to use the exact parental sensitive cell line from which the resistant line had been developed as the appropriate control in each CGH analysis.

Cisplatin resistance was examined in the cell lines CH1, 41M, and A2780. The CGH ratio profiles for these cell lines with acquired resistance to cisplatin, compared with their sensitive 'parental' cell lines, are shown in Figure 2. Each of these cell lines shows changes in copy number of discrete regions of DNA during the acquisition of drug resistance. However, few of the changes are common between these cell lines, with the notable exception of the gain of 6q in 41M and A2780 and the gain of 4q and 7p in CH1 and 41M.

Overall for the three different platinum analogues in three different parent cell lines, we have shown the most frequently observed changes to include gain of 4q (5/7) and 6q (5/7), followed by gain of 5q21–23 (3/7). The minimal common overrepresented region (MCOR) on chromosome 4 was q23 in 4/7 lines and q26–27 also in

4/7 cell lines. Likewise, the MCOR on chromosome 6 was q21–23 in 4/7 lines and q25–27 also in 4/7 cell lines. Thus, two different sites on 4q and 6q may be important loci for genes involved in platinum analogue resistance. A summary of amplified and deleted regions, acquired with resistance to the three different analogues, is illustrated with chromosome ideograms in Figure 3.

In terms of chromosomal aberrations associated with specific analogues, for the more lipophilic analogues, gain of 3p occurs only in the two cell lines resistant to JM216 (Figure 1, B and C), and deletions of 17p and Xp occur only in the two cell lines resistant to AMD473. Gain of 12p was seen only in the CH1 cell lines with acquired resistance (Figure 1B). The 41M-sensitive cell line showed high level amplifications of 3q, 9p, and 12p when compared with normal DNA. With the acquisition of resistance to cisplatin, some of these extra copies were lost from chromosomes 3q and 9p; the acquisition of resistance to JM216 was accompanied by loss of extra copies from 9p only. However, the high-level amplification of 12q was retained in both the JM216 and AMD473 resistant cell lines; this phenomenon of equal amplification in sensitive and resistant cell lines is illustrated in Figure 1C as a bright yellow region on the chromosome, with no alteration in the ratio profile.

Conclusions

A steadily increasing number of specific chromosomal abnormalities are found to be associated with particular tumor subtypes. In leukemias, these usually involve specific translocations, whereas in solid tumors they frequently include extensive rearrangements. These cytogenetic abnormalities define the sites of specific genes whose alteration is implicated in the neoplastic process. High level amplifications occur frequently in solid tumors and are often manifested cytogenetically in the form of homogeneously staining regions and double minute chromosomes.

The true incidence and significance of solid tumor chromosome abnormalities are limited in at least three different ways. First, these investigations are often performed late in the disease on samples from effusions or metastases, when the karyotype may be dominated by secondary abnormalities acquired during progression. Second, the chromosome preparations obtained from solid tumors are usually of poorer quality than those obtained from bone marrow or peripheral blood. Third, the majority of karyotypes obtained from solid tumors contain marker chromosomes in which the contributing chromosomes cannot be identified. The advent of CGH has enabled investigators to overcome most of these limitations.

The use of CGH and molecular cytogenetic techniques in the study of drug resistance has received little attention to date. Hoare et al used reverse *in situ* hybridization to detect a new amplicon in the doxorubicin-resistant lung cancer cell line, GLC4-ADR, which mapped to chromosome 1. They concluded that these techniques were ideally suited to characterizing genetic changes specific to drug resistance within a background of genetic anomalies associated with tumor progression.[9] This work builds upon prior publications by Kallioniemi and Tanner, who used CGH to screen 15 breast cancer cell lines and 33 primary tumors to identify and map regions of the genome with increased DNA-sequence copy number.[6] The majority of 26 chromosomal subregions involved did not correspond to the loci of currently known amplified genes in breast cancer with the highest frequency observed in 17q22–24 and 20q13. They subsequently studied the area of 20q13 by interphase FISH with anonymous cosmid probes and gene-specific P1 clones. They narrowed the critical region of interest to approximately 1.5 mb at 20q13.2. Previously known genes were excluded as candidates,[22] implying that this chromosomal region harbors a novel oncogene that contributes to the malignant progression of breast cancer. This demonstrates that CGH can be used to search directly for novel oncogenes associated with tumor progression and should be equally applicable to the search for novel oncogenes associated with specific resistance pathways.

In 1997, Wasenius et al[23] used CGH to look at cell lines with acquired resistance to cisplatin and acquired resistance to antimony and arsenite cross-resistant to cisplatin. In agreement with our work, they observed only low-level amplifications. They reported regions of 9 chromosomes to be frequently associated with resistance to cisplatin, of which the most frequently observed change was loss of 2p and gain of 2q (3/4 cell lines resistant to cisplatin). In this study we benefited from the extensive bank of ICR cell lines in which we were able to examine 7 cell lines resistant to 3 platinum analogues. Our results have demonstrated that gains of material on chromosome arms 4q and 6q are the most consistently observed changes and potentially the most significant (these were among the changes noted by Wasenius et al). The MCORs that we have identified on 4q and 6q are broadly consistent with the areas identified by Wasenius et al.

We also report the following chromosomal aberrations associated with specific analogues in the context of their biochemical mechanism of action.

Cisplatin

It is interesting to note that for cisplatin, the chromosomal aberrations observed in the three resistant cell lines differ substantially from each other. Previous biochemical studies confirm that the mechanisms of resistance differ significantly in these three cell lines. Kelland, Harrap, and others have shown that resistance in the 41M cell line is predominantly mediated by a transport mechanism,[12] whereas increased DNA repair and/or tolerance of DNA-platinum adducts is the major mechanism of resistance in the CH1 cell line.[10] Reduced drug accumulation, elevated glutathione levels, enhanced capacity to remove platinum adducts, and failure to engage the appropriate apoptotic response all play a role in the acquired resistance of the A2780 cell line.[11]

JM216

In contrast, the platinum analogue JM216 is sufficiently lipophilic that transport does not play any significant role in the development of acquired resistance in the 41M cell line. Hence, one would presume that the CH1 and 41M cell lines share similar mechanisms of resistance to JM216. Our CGH results show overrepresentation of the same region of chromosome 3p for both CH1 and 41M cell lines made resistant to JM216. It is interesting that this region encompasses the sites of the MLH-1 DNA repair gene and topoIIβ.

AMD473

The sterically hindered platinum analogue AMD473 has a lipophilicity between that of cisplatin and JM216. The mechanisms of resistance to AMD473 have been shown to include reduced accumulation, reduced DNA platination, and increased capacity to forgo apoptosis in both the CH1 and A2780 cell lines.[24] Our CGH results show a unique loss of copies of the regions Xp and 17p associated only with AMD473 resistance.

Although these associations of chromosome abnormalities with potential mechanisms are thought-provoking, they should not be overinterpreted in light of the small number of cell lines. There is a degree of cross-resistance between cell lines with acquired resistance to JM216 and AMD473, as well as to cisplatin itself. Thus,

changes in common among cell lines with acquired resistance to the different analogues would be expected, corresponding to the sites of genes involving shared mechanisms of resistance, such as the changes on 4q, 5q, and 6q.

The classically quoted biochemical pathways associated with cisplatin resistance (decreased intracellular accumulation, elevated glutathione, elevated metallothioneins, enhanced DNA repair, altered mitochondrial membrane potential, increased oncogene expression, and signal transduction) were summarized by Marshall and Andrews[25]; multiple publications have confirmed either protein and/or mRNA changes in these parameters.[26,27] In the majority of cell lines, cisplatin resistance appears to be multifactorial. A summary of specific genes which have been associated with resistance to cisplatin and their chromosomal location is presented in Table 2. It is unclear whether these specific genetic changes are primary or secondary. A recent paper by Anthoney and Brown[56] has demonstrated the appearance of microsatellite alleles at multiple loci in resistant lines, suggesting an association between selection for cisplatin resistance and the development of genomic instability.

In summary, our results comparing seven cell lines and three agents have shown the most frequently observed changes to involve amplification of 4q(5/7) and 6q (5/7), followed by amplification of 5q (3/7). These common areas of amplification and deletion across different cell lines in response to the acquisition of resistance to the same drug or analogues indicate potential sites of genes involved in common mechanisms of drug resistance. These aberrations include the sites of EGF, HMG2, and cyclin B, all of which have been previously implicated in cisplatin resistance. Other candidate genes in these regions include MSH3 (5q11–12), APC (5q21–22), and DHFR (5q11–13), which are all involved in DNA repair; the genes cyclin C (6q21), CD24 (6q21), and PDCD2 (programmed cell death 2) (6q27), involved in signal transduction; and GST2 (4q28–31), the glutathione s-transferase gene.

Some aberrations were induced only in response to a specific analogue. These included the amplification of 3p (site of both the topoIIβ and MLH1 genes) seen in response to acquired resistance to JM216; deletions of 17p (site of p53) and Xp (site of CLCN4 and POLA) seen only in response to acquired resistance to AMD473. These areas may include genes involved with a more selective mechanism of resistance. The regions that we have identified are currently being investigated by FISH using specific gene probes to narrow the critical region and identify candidate genes.

These cell line data have provided insight into the association of defined chromosomal abnormalities with specific mechanisms of resistance. It is now critical to extend this work into tumor biopsies obtained from patients. Hence, we are now beginning a major study to analyze samples from patients with intrinsic and acquired resistance to the three platinum analogues that we have described.

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