Similar Chromosomal Changes in Cisplatin and Oxaliplatin-Resistant Sublines of the H69 SCLC Cell Line are not Associated with Platinum Resistance

Britta Stordal[1], Greg Peters[2] and Ross Davey[1*]

[1]Bill Walsh Cancer Research Laboratories, Royal North Shore Hospital, St Leonards, 2065 NSW, AUSTRALIA.
[2]Department of Cytogenetics, The Children's Hospital at Westmead, Westmead, 2145 NSW, AUSTRALIA.

[*]**Correspondence to:**

Adjunct Prof Ross Davey
Bill Walsh Cancer Research Laboratories
Royal North Shore Hospital
St Leonards NSW 2065
AUSTRALIA

Tel: +61-2-9926-7456
Fax: +61-2-9926-5253
email: rdavey@med.usyd.edu.au

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Small cell lung cancer (SCLC) initially responds well to DNA damaging drugs such as cisplatin, however this is transitory as resistance normally develops. To investigate whether changes in chromosomal copy number caused by platinum drug treatment contributes to platinum resistance; we have analysed H69 SCLC cells and two low-level platinum-resistant sublines, H69CIS200 and H69OX400, derived by cisplatin and oxaliplatin treatment respectively. Affymetrix 10K SNP array showed that cisplatin and oxaliplatin have independently caused similar changes including loss of segments 6q21-qter and 13pter-13q.14.11 and duplication of chromosome 21. Interestingly, despite using equally cytotoxic doses of drug in the development of the cell lines, oxaliplatin caused three times more chromosomal changes than cisplatin. The resistant cell lines lose their resistant phenotype after 3 months of drug-free culture. The revertant cell lines, denoted H69CIS200-S and H69OX400-S, were also analysed by Affymetrix array to determine if chromosomal changes associated with resistance remain after the resistant phenotype is lost. In the H69OX400-S many of the changes observed in the resistant cells were absent suggesting that they contributed to the resistant phenotype including: loss of 1q23.3-qter, 10q11.23 and 19q13.12-q13.2 and duplication of segments 6p21.2-p12.3, 16q12.1-16q13, 16q21-q23.1 and 19q12. However, out of the similar changes induced by cisplatin and oxaliplatin, both the loss of 6q21-qter and gain of 21 were still present in the H69CIS200-S and H69OX400-S cells. This suggests that cisplatin and oxaliplatin induced similar changes due to inherent vulnerabilities in the H69 cells rather than changes associated with platinum resistance.

INTRODUCTION

The karyotypic abnormalities of drug-resistant cells have been studied extensively with the hope of gaining insight into the drug-resistant phenotype. Changes in gene copy number at loci associated with drug resistance have provided links between changes in the genotype of a cell line and its resistant phenotype. These changes have also provided clinical markers used to monitor the development of resistance over the course of treatment. The increase in gene copy number for ABC transporters *ABCC1* (MRP1) at 16p13.1 (Cole et al., 1992) and *ABCB1* P-glycoprotein (P-gp) at 7q36 (Bell et al., 1987) have been associated with resistance to doxorubicin and vinblastine respectively. The increase in copy number of *DHFR* (dihydrofolate reductase) has also been associated with the development of methotrexate resistance in human and animal cell lines (Fougere-Deschatrette et al., 1984) as well as clinically in the treatment of leukemia (Horns, Jr. et al., 1984).

Chromosomal rearrangements have been studied in many cisplatin-resistant cancer cell lines (Table 1) in the hope of discovering a unique marker of platinum resistance. Many rearrangements have been observed, including amplifications and deletions on almost every chromosome, as summarised in Figure 1. There are more amplifications (57.4%) than deletions (42.6%) associated with cisplatin resistance, and the number of changes in a cell line does not correlate with the level of cisplatin resistance. Chromosomal changes have also been studied in tumour samples from patients who have received platinum-based therapy with a similarly large array of changes found (Rao et al., 1998; Kudoh et al., 1999; Cullen et al., 2003). Chromosomal changes have not been previously studied in cisplatin-resistant small cell lung cancer (SCLC) or in oxaliplatin-resistant cell lines.

Very few links to the platinum-resistant phenotype have been established by examining the changes in chromosomes. A wide ranging study examining many types of tumours resistant to several drugs, looked for changes in copy number at known drug resistance loci. It established several links to the resistant phenotype for etoposide and

camptothecin but not for any of the cisplatin-resistant cell lines examined (Yasui et al., 2004). To our knowledge, the only study which has successfully linked changes in genotype to the platinum-resistant phenotype involved an amplification of 11q13 and overexpression of *GSTP1* which was associated with cisplatin resistance in tumours of the head and neck in both cell lines and tumour biopsies (Cullen et al., 2003).

A large number of chemotherapeutics including platinum drugs achieve cytotoxicity through DNA damage. The platinum drug binds to the DNA strand hindering DNA transcription and replication and sometimes causing chromosome breakage. The chromosomal aberrations caused by this DNA damage are thought to be non-random in their genomic distribution (Meyne et al., 1979). Platinum drugs bind to GC-rich areas but not specific DNA sequences. Therefore, it is not clear which regions of the genome are more susceptible to damage. Chromosomal aberrations can in theory lead to the development of a drug-resistant phenotype. However, most chromosomal changes caused by DNA damaging drugs will not involve loci associated with drug resistance and will not contribute to the resistant phenotype. Amplifications and deletions can often arise in a single exposure to drug, and it is difficult to determine if it is the amplification of one gene or the loss of another that is responsible for resistance. Chromosomal changes accumulated in response to treatment with platinum drugs could be a part of the mechanism of drug resistance or they could be nothing more than a by-product of exposure to DNA damaging chemotherapeutics and not responsible for the resistance.

The contribution of any particular chromosomal change to the resistant phenotype is difficult to study. However, we believe that our cellular model of platinum drug resistance may provide a unique insight. We have developed a clinically relevant model of low-level cisplatin and oxaliplatin resistance in human H69 SCLC cells (Stordal et al., 2006). The H69CIS200 and H69OX400 cells are stably 2-fold resistant and cross-resistant to both platinum drugs for 6-8 weeks in drug-free culture. After this period their resistant phenotype fades in the absence of drug as determined by an MTT cytotoxicity assay. We have designated these drug-sensitive revertant cell lines H69CIS200-S and H69OX400-S.

We have performed an Affymetrix 10K SNP array and cytogenetic analysis to characterise the chromosomal changes in these resistant cell lines during their period of stable resistance and again after the resistant phenotype spontaneously faded. Through this novel approach we hope to demonstrate which rearrangements are associated with the drug resistance phenotype and which are random DNA damage. This study is the first SNP array analysis of the H69 SCLC cell line. This is also the first SNP array and cytogenetic analysis of either a cisplatin-resistant SCLC cell line or an oxaliplatin-resistant cell line of any carcinoma.

METHODS

Cell Culture

The human H69 small cell lung cancer cell line was obtained from the American Type Culture Collection. The H69CIS200 and H69OX400 cells were developed over 8 months with 8 4-day treatments of 200 ng/ml cisplatin and 400 ng/ml oxaliplatin respectively (Stordal et al., 2006). These cell lines were stably 1.5 to 2-fold resistant for approximately 6-8 weeks in drug-free culture. Cisplatin IC_{50} H69 – 1.24 μg/ml, H69CIS200 – 2.11 μg/ml, H69OX400 – 2.14 μg/ml. Oxaliplatin IC_{50} H69 – 2.62 μg/ml, H69CIS200 – 4.17 μg/ml, H69OX400 – 4.71 μg/ml. The resistant phenotype fades over the next 6-8 weeks in drug-free culture. The revertant cell lines were designated H69CIS200-S and H69OX400-S which both have the same IC_{50}s as the parental cell line. There was no change in growth rate or morphology associated with the gain or loss of resistance. All cells and sublines were maintained in drug-free RPMI (Thermoelectron, Sydney, Australia) with 10% FCS in a humidified atmosphere with 5% CO_2 at 37°C. The H69 cells and sublines have not been cloned and all cultures were *mycoplasma* free.

MTT Cytotoxicity Assays

To determine the level of resistance, cells were plated into flat bottomed 96-well plates at a cell density of 6.0 x 10^4 cells/well. Cells were treated in triplicate with 2-fold serial dilutions of drug in a final volume of 200 µl. Drug free controls were included in each assay. Plates were incubated for 5 days at 37°C in a humidified atmosphere with 5% CO_2 and cell viability was determined using the MTT assay. 50 µl of MTT (2.5 mg/ml in PBS) was added to each well and the cells incubated for a further 2 hours. The plates were centrifuged at 800g for 5 minutes, the culture medium aspirated and the formazan product dissolved in 100 µl DMSO. Plates were mixed for 15 minutes and the absorbance measured at 570 nm. Cell viability was calculated as a percentage of control absorbance values and the fold resistance was calculated by dividing the IC_{50} of the resistant cells by that of the H69 cells.

Affymetrix 10K SNP Array

Genomic DNA was extracted from the cell lines using Qiagen Genomic Tips according to the manufacturer's instructions. The Affymetrix genechip mapping 10K array Xba 131 was performed by the CSIRO Department of Molecular and Health Technologies, Sydney, Australia. The results of the array were then analysed with the Affymetrix chromosomal copy number tool. The Affymetrix system compares the chromosomal copy number tool data to a pool of normal samples in order to determine SNPs that have changed. We had to analyse our data differently to compare our cytogenetically abnormal samples to each other. The resistant and revertant samples were compared to the drug-sensitive parent by the following method. The chromosomal copy number data for each chromosome was smoothed by averaging a window of 20 SNPs. Significant differences, indicating amplifications or deletions were defined as any smoothed region which had at least 3 SNPs in a row in which the resistant cell line differed from that of the parental by a gene copy number of at least 0.5.

Cytogenetics

Metaphase chromosome spreads were prepared using standard cytogenetic protocols with the following modifications. More colcemid was used than usual (3.33 mg/mL) and the hypotonic shock of KCl was 0.03M. Trypsin-Giemsa banding was performed using standard cytogenetic protocols (Barch, 1991). A combination of commercial and 'in house' FISH probes were used in this project and these are summarised in Table 2. The preparation of probes from plasmid DNA and the FISH experiments were performed using previously published protocols (Daniel et al., 2004).

RESULTS

H69 Parental Cell Line

The H69 SCLC cell line was analysed by an Affymetrix 10K SNP array. This revealed many changes in copy number associated with the cancerous phenotype. The highest copy number amplifications were on chromosome 8 and 14 (Figure 2A). The H69 cell line and all its sublines were quasi-tetraploid, each cell having around 60 chromosomes per cell, including 3 structurally normal copies of chromosome 8 (Whang-Peng et al., 1982). The gross amplification of chromosome 8 sequences was confirmed to be the *MYC* gene by FISH (Figure 2B). Cytogenetically, the *MYC* amplification was seen within a homogenously staining region located on a derivative fusion chromosome much larger than chromosome 1. There were also 3 copies of *MYC* on normal copies of chromosome 8 (circled). The *MYC* amplification within the large chromosome was flanked by a copy of chromosome 15 providing the centromere and one telomere. The other end of this derivative chromosome is a copy of 6q21-qter providing a second telomere. This *MYC* amplification and the large undefined amplifications on chromosome 14, did not alter with the development of drug resistance as both were present at the same level of amplification in the H69CIS200 and H69OX400 resistant sublines.

H69CIS200 and H69OX400 Resistant Cell Lines

The platinum-resistant cell lines had many genotypic changes from the parental cell line as determined by the Affymetrix 10K SNP array (Figure 3). The greatest number of chromosomal aberrations occurred on chromosome 6 (Figure 4A) and this chromosome was therefore further examined by FISH. Some changes observed were very similar in the H69CIS200 and H69OX400 cells despite their independent treatments with different platinum drugs. The similarities were a deletion of segments 6q21-qter and 13pter-13q14.11 and duplication of chromosome 21 (Figure 3). There was a greater number of smaller changes due to oxaliplatin treatment than due to cisplatin treatment.

The duplication of segment 6q12-q21 and deletion of 6q21-qter were further examined in the H69CIS200 cells. Figure 4B shows a single-locus FISH probe RP11-124K9 at 6q15 in a representative H69CIS200 metaphase. The 6q15 probe showed 2 separate regions of labelling (green) as marked in Figure 4B and were i) a normal copy of chromosome 6 and ii) a tandem duplication of 6q15. The red probe is a 6p sub telomere control for chromosome 6. In contrast, the H69 parental cells and the H69OX400 cells showed normal dosage for 6q15 on both copies of chromosome 6 (data not shown).

The chromosome 6 paint of the H69CIS200 cells showed 4 separate regions of labelling (Figure 4C). As marked on Figure 4C these were i) a normal copy of chromosome 6, ii) a larger copy of 6 with 6q12-q21 duplication and fused to another chromosome at 6q21, iii) another copy of the segment 6q21-qter as part of the large *MYC* derivative chromosome described earlier and iv) a small segment of 6p forming one end of a small metacentric chromosome.

The duplication in 6p in the H69OX400 cells was examined using single locus probes for RP11-262E12 at 6p21.2 (green) and RP11-876F11 at 6p12.3 (red) which were bands corresponding to the peak of the amplifications as identified by the Affymetrix SNP array. The amplification in the H69OX400 cells could not be detected in the metaphases seen via FISH but could be observed in 26.5% of interphase nuclei on the

same slide (Figure 4D). This indicates mosaicism for this abnormality. Figure 4E shows two interphase cells from the H69OX400 cell line. The cell on the left has three copies of the 6p21.2 and 6p12.3 probes, whereas the cell on the right has the normal two copies. The additional copy was not detectable in either the parental H69 cells or the H69CIS200 cells, showing that this duplication was specific to the H69OX400 cells as predicted from the Affymetrix analysis.

The loss of heterozygosity (LOH) analysis revealed a LOH in regards to the 6q21-qter deletion for the H69CIS200 cells but not for the H69OX400 cells. The LOH analysis is limited in its power to detect changes between the H69 cells and the resistant cell lines because of the large amount of LOH already present in the H69 cells compared to a normal non cancerous sample. The Affymetrix array gives a call of AA, AB or BB for each SNP. A shift from an AB call to an AA or BB indicates a LOH. This can mean the second copy has been lost and the locus is hemizygous. Alternatively, in a polyploid cell line such as the H69 cells, it can mean that one of three copies has been lost and the two that remain are of the same parental origin. A LOH can also occur if DNA damage has occurred in a locus and it has been repaired by referring to the other copy. The H69 cells have AB calls for only 12.33% of the SNPs assayed in the 10K array. This is quite low compared to normal non cancerous samples which usually have 30% of the genome as AB calls (Wong et al., 2004). The AB calls for the H69CIS200 cells is 11.51% and this difference is primarily due to the LOH associated with the deletion of 6q21-qter. The AB calls for the H69OX400 cells remains similar to the parental H69 cell line at 12.35%.

H69CIS200-S and H69OX400-S Sensitive Revertant Cell Lines

The H69CIS200 and H69OX400 cells were stably 2-fold resistant and cross-resistant to cisplatin and oxaliplatin for 6-8 weeks in drug-free culture as determined by an MTT cytotoxicity assay. Over a further 6-8 weeks their resistant phenotype fades (data not shown), and we have denoted these revertant cell lines H69CIS200-S and H69OX400-S.

The H69CIS200-S and H69OX400-S cell lines were analysed by Affymetrix array. We also analysed H69 parental cells that were grown over the same 3-month period during which the revertant cell lines were losing their resistance. The H69 parental cell line sampled here showed very similar results to the first experiment, indicating that the chromosomes of this cell line were stable in drug-free culture. However, the H69CIS200-S and H69OX400-S sensitive cells showed many differences from the resistant cell lines from which they were derived. Figure 5A shows the copy number profile of chromosomes 1, 6, 10, 13, 16, 19, 21 and X in both the drug-resistant and revertant drug-sensitive cell lines. Some of the changes developed with resistance are absent in the revertant cell lines suggesting that these changes may contribute to the resistant phenotype. Figure 5B summarises the chromosomal changes that were lost with the loss of resistance and are therefore associated with the resistant phenotype. Figure 5C summarises those changes still present with the loss of resistance and are therefore not associated with the resistant phenotype.

The amplification of 6q12-q21 in the H69CIS200-S cells increased with the loss of the resistant phenotype. The H69OX400-S cells also had two other segments of change associated with the loss of resistance, duplication of 4q34.3-qter and 12pter-12p13.31 (data not shown). However, these regions are not associated with the mechanism of resistance as these changes arose in conjunction with a loss rather than a gain of resistance. This data suggests that the genome of the resistant cells was more dynamic than that of the parental cell line which had no changes over the 3 month culture period.

The H69CIS200-S and H69 parental cells grown for 3 months in drug-free culture showed no major changes in LOH compared to the original analysis (11.70% and 12.40% AB calls respectively). However, the H69OX400-S cells dropped their AB calls to 11.83%. This is due in part to a new region of LOH at 6q21-qter not associated with a change in copy number. This is the same region which showed LOH with the deletion of a copy in the H69CIS200 cells. Clearly this is a very unstable region in this cell line. It is possible that the H69OX400-S cells experienced some damage to, or a complete deletion of, this region over the 3 months in culture and repaired it by duplicating a remaining

copy without altering the copy number for this region. Hence a new region of LOH arose without a change in gene copy number.

DISCUSSION

The H69 SCLC cell line is a near-tetraploid cell line with up to 87 chromosomes per cell (Whang-Peng et al., 1982). The *MYC* amplification in SCLC has been shown previously by Affymetrix 10K SNP array (Zhao et al., 2004) but has not been visualised by FISH before (Figure 2B). In vivo, overexpression of *MYC* leads to uncontrolled cell growth and proliferation, and so this amplification most likely occurred in vivo before the cell line was established. This probably contributed to the cancerous phenotype of the original tumour. The *MYC* homogenously staining region is flanked by an almost complete copy of chromosome 15 including the centromere and a copy of segment 6q21-qter. These segments of 15 and 6q21-qter confer stability to this large derivative chromosome but are unlikely in themselves to contribute to the cancerous phenotype.

The large amplifications on chromosome 14 did not alter with the development of drug resistance, but could have been associated with the initial cancerous phenotype. Amplifications in the region 14q12-q21 were also found in other SCLC cell lines but any specific association with malignancy is unknown (Ashman et al., 2002). The H69 cells also had a loss of 3p which is characteristic of SCLC cells (Whang-Peng et al., 1982).

The Affymetrix 10K SNP array (Figure 3) revealed many similar changes in both resistant sublines relative to the parental sensitive cell line. The question remains as to whether these similarities are due to the similar nature of the two chemotherapeutics used in drug treatment or because these breakpoints are places of inherent vulnerability in the parental cell line.

There were more changes in the oxaliplatin-resistant cell line than in the cisplatin-resistant cell line even though the doses used in development were equivalent in cytotoxicity. Cisplatin and oxaliplatin form the same types of DNA adducts at the same

sites on the DNA strand (Woynarowski et al., 1998; Chaney et al., 2005) which may explain some of the similarities in chromosomal changes shared by our resistant cell lines. However, oxaliplatin is more cytotoxic per DNA lesion than cisplatin (Woynarowski et al., 1998); which may in part explain why there was an increase in chromosomal rearrangement in the oxaliplatin-resistant cell line in comparison to the cisplatin-resistant cell line.

The cisplatin and oxaliplatin-resistant cell lines both have a breakpoint at 6q21 and a subsequent deletion of 6q21-qter. The H69 parental cell line has a duplication of the same region (6q21-qter) seen on the derivative chromosome carrying the *MYC* amplification. Therefore the resistant cells have copy numbers of chromosome 6 that are closer to that of a normal cell than their drug-sensitive parent. The LOH analysis also revealed that the loss of 6q21-qter was on different copies of chromosome 6 (differing in respect of parental origins) in the two resistant cell lines. Similarly, a cisplatin-resistant ovarian carcinoma and its parental cell line (A2780/A2780CP8) both share changes in 6q21. The parental has a translocation with 1q23 and the resistant cell line has a deletion of 6q21 (Behrens et al., 1987). This suggests that if a chromosomal region is altered in the process of oncogenesis it may be altered again in the development of drug resistance. There are also several other studies which have reported a "correction" of the genome with cisplatin resistance (Walker et al., 1990; Yasuno et al., 1999), where amplifications in the parental cell line are deleted in the resistant subline.

Cisplatin-induced DNA damage in normal cells is to some extent non-random, having 'hot spots' where damage is most likely to occur (Meyne et al., 1979). The preferential assignment of chemically induced breakpoints to lightly staining G-bands has also been reported with particular reference to the junction point between a light and dark G-band (Meyne et al., 1979). However, this could also be due to systematic observer bias as G-band junctions are easier to see. The chromatin within certain bands may be more prone to damage by a variety of DNA damaging chemical agents (Brogger, 1977), including the lightly staining band 6q21 of interest in this study. There are many changes on chromosome 6 observed in other cisplatin-resistant cell lines, summarised in Figure 1.

In the region 6q21-qter most changes previously reported are duplications rather than deletions as found in this study. However, the large number of chromosomal changes around 6q21 seen in many studies suggest this region is a 'hot spot' for chromosomal breakage due to platinum.

There are several possibilities to explain why a particular region may be more prone to damage and breakage from treatment with platinum. The region may be particularly GC-rich which means a platinum adduct is more likely to occur. The presence of fragile sites or a loss in chromatin methylation may also render a region more susceptible to DNA damage. The GC content of the regions associated with chromosomal breakage in our resistant cell lines was analysed using the GC content maps available at http://genomat.img.cas.cz. (Paces et al., 2004). While breakages tend to occur in regions of GC content 40% or greater they do not occur at the most GC-rich areas of the genome. Chromosomes 16, 17, 19, 20 and 22 are very GC-rich and it is clear from the summary of breakage due to platinum in the literature (Figure 1), and the results of this study, that these chromosomes are some of the least damaged by platinum. High GC-rich regions are also the regions containing the most genes and when transcriptionally active are more open to chemical attack. Perhaps some mechanisms of DNA repair such as transcription-coupled nucleotide excision repair, which repairs actively transcribing genes (Sancar et al., 2004), efficiently repairs the damage to these GC-rich small chromosomes.

The mechanism of loss of resistance of a cell line has not been studied extensively. Some resistant cell lines are stable for long periods in drug-free culture (Slovak et al., 1993), some require the presence of drug either constantly (Burns et al., 2001) or regular short treatments to maintain their resistant phenotype (Locke et al., 2001). The H69CIS200 and H69OX400 cell lines revert to a drug-sensitive phenotype as determined by MTT assay after 3 months in drug-free culture (data not shown). By analysing the chromosomes of the drug-sensitive revertant cell lines, denoted H69CIS200-S and H69OX400-S, we can determine if the chromosomal changes observed in the resistant cell lines were associated with the resistant phenotype. There are two broad hypotheses for what could be causing a loss of the resistant phenotype in our

cell models. The first hypothesis is that there is a homogenously resistant population and the loss of the resistant phenotype is due to a downregulation of stress response pathways as the cells grow for many weeks in drug-free culture leading to a sensitive phenotype. The chromosomal changes previously detected in the resistant cell line will still be present and were not part of the mechanism of resistance. Alternatively, the second hypothesis is that there is a mixed population of resistant and sensitive cells. Initially the resistant cells dominate as the population has been recently exposed to a chemotherapeutic, as weeks pass in drug-free culture the sensitive cells dominate the culture as they grow faster. This may be due to the taxing nature of the resistance mechanism on cell growth. The chromosomal changes previously detected will be absent as the sensitive cells without the changes associated with the resistance mechanism have dominated the culture.

As far as we could tell, all chromosomal changes seen in the H69CIS200 cells were non-mosaic as they occurred in all metaphases examined. This suggested a homogenously resistant population of cells. From the results of the Affymetrix array on the sensitive revertant cells the H69CIS200 cells appear to fit the first hypothesis. All the chromosomal changes detected in the resistant cell lines were still present in the sensitive revertant cells. A downregulation of stress response pathways is the most likely explanation for the loss of the resistance mechanism. The chromosomal changes may have been involved in the development of resistance but their presence alone is not sufficient to maintain the resistant phenotype. The transcription of key genes is likely to be required to maintain the mechanism of drug resistance. The loci containing these genes may have been amplified or experienced changes in DNA methylation in association with the development of drug resistance. However, without transcription the resistance mechanism will be lost. The mechanisms of platinum resistance in these cell models are currently being investigated and will be examined for any association to the chromosomal changes observed.

The H69OX400 cells were generally non-mosaic but were mosaic for the 6p duplication as it was detected in 26.5% of interphase nuclei (Figure 4D). This suggests a

mixed population of cells in the H69OX400 cell line. The H69OX400 cells appear to fit the second hypothesis as many of the changes associated with the resistant phenotype are absent in the revertant cell lines. This suggests that these particular chromosomal changes, combined with the upregulation of stress response pathways, were part of the resistance mechanism. However, three changes did not revert in the H69OX400-S cells, the loss of segments 6q21-qter and Xp and gain of chromosome 21. These changes were therefore not associated with the mechanism of resistance.

It is interesting to note that out of the three large changes that were seen in both the cisplatin and oxaliplatin-resistant cell lines, two of them (loss of the segment 6q21-qter and gain of chromosome 21) were not associated with the mechanism of resistance (Figure 5C). The other large change, loss of segment 13pter-13q14.11, was associated with oxaliplatin resistance but not cisplatin resistance (Figure 5B and C). This suggests that these regions are inherently vulnerable in H69 cells and are not part of the mechanism of platinum resistance. Small genomic alterations beyond the power of detection of the Affymetrix 10K array may also be associated with resistance. However, the large chromosomal aberrations in common between the H69CIS200 and H69OX400 cells seem not to be associated with platinum resistance.

The reversion of chromosomal changes associated with the loss of resistance has been shown previously in H69 cells resistant to doxorubicin. Chromosomal amplification of MRP1 (16p13.1) in the form of both a homogenously staining region and double minute chromosomes led to doxorubicin resistance in H69AR cells (Cole et al., 1992). However, these MRP1 double minutes were no longer present in H69PR cells which had partially reverted to a drug-sensitive phenotype over 36 months in culture (Slovak et al., 1993). This demonstrates that a resistant cell line grown in culture without the selective pressure of drug can revert to a more drug-sensitive phenotype and genotype. The authors used this as further evidence for the amplifications being responsible for the drug-resistant phenotype. The authors did not speculate on the reasons for the reversion to a sensitive phenotype. It seems likely that the maintenance of the MRP1 amplification in the double minutes became a burden for the resistant cells. However, it should be noted

that double minutes are a special case as they lack centromeres and are spontaneously lost in the absence of selective pressure from drug treatment. The H69PR cells seem to be an example of the second hypothesis, like our H69OX400 cells, where with time without selective pressure a more sensitive sub-population dominates the culture.

Another study which showed a small amplification in the gene for P-gp in a doxorubicin-resistant sarcoma reported interesting results for their revertant cell lines. Their revertants were selected for by flow cytometry-sorting for high or low P-gp expression. The revertant cell line had low-level P-gp expression but carried the same amplifications observed in the parental cells (Chen et al., 2002). This indicates that the amplification alone was not sufficient to produce resistance in these cell lines, upregulation of the transcription of the gene was also required. This is similar to our H69CIS200 cells, where the presence of chromosomal change alone was not enough to maintain resistance, the upregulation of the stress response was also required to maintain resistance.

Cisplatin and oxaliplatin promote genomic rearrangement in low-level platinum-resistant SCLC cells. Oxaliplatin is better than cisplatin at promoting chromosomal change and changes that may be associated with resistance. However, the major changes in common between the two platinum drugs seem not to be associated with resistance, but rather reflect the vulnerability of the genome to platinum drugs. Many studies have found the same karyotypic abnormalities in multiple cisplatin-resistant cell lines and have logically concluded that these changes were part of the resistant phenotype. However, from our search of the literature there does not appear to be any phenotypic link between 'hot spots' of chromosomal change due to platinum and platinum resistance. Our study suggests that finding similar chromosomal changes in multiple platinum-resistant cell lines may not reflect the platinum-resistant phenotype. These 'hot spots' must therefore be viewed with caution when considering potential markers for the diagnosis of clinical platinum resistance.

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Figure Legends

Figure 1 – Summary of chromosomal changes previously associated with cisplatin resistance in cell lines. Lines to the left indicate a deletion, lines to the right indicate an amplification. Papers used in the preparation of this diagram are presented in Table 1.

Figure 2 – Analysis of the H69 human small cell lung cancer cell line. A) Affymetrix chromosomal copy number analysis for Chromosomes 8 and 14. Genomic DNA from H69 cells was analysed with an Affymetrix 10K SNP array and Affymetrix chromosomal copy number tool. B) H69 metaphase stained with *MYC* FISH probe (red). Metaphase chromosomes stained with *MYC* (red) reveal a large *MYC* amplified region and 3 normal copies of *MYC* on chromosome 8 indicated with circles.

Figure 3 - Chromosomal copy number changes in the resistant cell lines. Genomic DNA from the H69CIS200 (——) and H69OX400 (——) cells were analysed with an Affymetrix 10K SNP array and Affymetrix chromosomal copy number tool and compared to the H69 parental cell line. Segments of change compared to the parental H69 cell line are presented, deletions to the left of the chromosome, amplifications to the right.

Figure 4 – Analysis of chromosome 6. A) Affymetrix chromosomal copy number of chromosome 6 in the H69 (——), H69CIS200 (——) and H69OX400 (——) cell lines. B) H69CIS200 metaphase stained with 6q15 probe (green indicated with arrows) and a 6p sub telomere control (red) reveal i) a normal copy of chromosome 6 and ii) an amplified copy of chromosome 6. C) H69CIS200 metaphase stained with chromosome 6 paint (green) reveal i) a normal copy of 6 ii) a copy of 6 with 6q12-q21 amplification, deletion of 6q21-qter and fusion to another chromosome iii) 6q21-qter as part of the large *MYC* chromosome iv) a piece of 6p as part of a metacentric chromosome. D) Number of copies of 6p probes in interphase nuclei. At least 100 interphase cells for each of the cell lines were scored for their number of 6p probes (□) 2 copies or (■) 3 copies and a

percentage calculated. E) Interphases from the H69OX400 cell line, the cell on the left has 3 copies of the 6p probes the cell on the right the normal 2 copies.

Figure 5 – A) Chromosomal copy number of chromosomes 1, 6, 10, 13, 16, 19, 21 and X showing the resistant cell lines H69CIS200 (——), H69OX400 (——) and the sensitive revertant cell lines H69CIS200-S (——), H69OX400-S (——). Chromosomal copy number changes were divided into two groups B) those associated with the mechanism of resistance and C) those not associated with the mechanism of resistance for the H69CIS200 (——) and H69OX400 (——) cell lines.

Table 1 – Cisplatin-Resistant Cell Lines Previously Analysed for Chromosomal Changes.

Cell Line	Cancer	Analysis	Cisplatin Resistance	References	Amp	Del
RT112-CP	Bladder	Karyotype	4.6	(Walker et al., 1990)	4	10
KK47/DDP10	Bladder	CGH	9.3	(Kotoh et al., 1997; Yasui et al., 2004)	4	1
KK47/DDP20	Bladder	CGH	18.7	(Kotoh et al., 1997; Yasui et al., 2004)	2	1
T24/DDP5	Bladder	CGH	2.2	(Kotoh et al., 1994; Yasui et al., 2004)	1	1
T24/DDP7	Bladder	CGH	5.2	(Kotoh et al., 1994; Yasui et al., 2004)	1	0
T24/DDP10	Bladder	CGH	8.4	(Yasui et al., 2004)	6	2
HT-29/cDDP	Colon	CGH	5	(Yamada et al., 1996; Yasui et al., 2004)	4	2
YES-2/CDDP	Esophageal	CGH	7.5	(Toshimitsu et al., 2004)	13	2
St-4/cDDP	Gastric	CGH	7	(Yamada et al., 1996; Yasui et al., 2004)	0	0
MeWo/Cis1	Melanoma	CGH	6	(Kern et al., 1997 ; Wittig et al., 2002)	2	3
BMI/BMIR2	Neuroblastoma	CGH	17	(Yasuno et al., 1999)	3	3
IMR/CP.17	Neuroblastoma	Karyotype	6.6	(Ireland et al., 1994)	1	1
SK/CP.12	Neuroblastoma	Karyotype	3.8	(Ireland et al., 1994)	4	0
2008/C8	Ovarian	CGH	4	(Wasenius et al., 1997)	9	5
2008/C13*5.25	Ovarian	CGH	15	(Wasenius et al., 1997)	11	9
2008/A	Ovarian	CGH	17	(Wasenius et al., 1997)	7	8
A2780/CP	Ovarian	CGH	11	(Wasenius et al., 1997)	0	5
CH1: CisR	Ovarian	CGH	6.4	(Leyland-Jones et al., 1999)	3	0
41M: CisR	Ovarian	CGH	4.7	(Leyland-Jones et al., 1999)	5	5
A2780: CisR	Ovarian	CGH	16	(Leyland-Jones et al., 1999)	3	2
KF28/KFr13	Ovarian	CGH	4.72	(Takano et al., 2001)	1	2
A2780/2780CP8	Ovarian	Karyotype	7.3	(Behrens et al., 1987)	0	3
GCT27cisR	Testicular	Microarray	5.6	(Kelland et al., 1992; Wilson et al., 2005)	4	8
833K/64CP	Testicular	Microarray	7	(Reilly et al., 1993; Wilson et al., 2005)	12	5
Susa-CP	Testicular	Microarray	4.2	(Walker et al., 1990; Wilson et al., 2005)	8	2
				Total	108	80

Table 2 - Probes Used for FISH.

Probe	**Preparation**	**Source**
6p21.2	Made from plasmid RP11-262E12	Cell & Gene Therapy Resource Unit, Murdoch Children's Research Institute, Parkville, Vic, Australia
6p12.3	Made from plasmid RP11-876F11	
6q15	Made from plasmid RP11-124K9	
6 Whole Chromosome Paint	Commercial	Cambio
6p sub-telomere	Made from plasmid 62I11	Incyte Genomics
C-myc (8q24.12-q24.13)	Commercial	Vysis

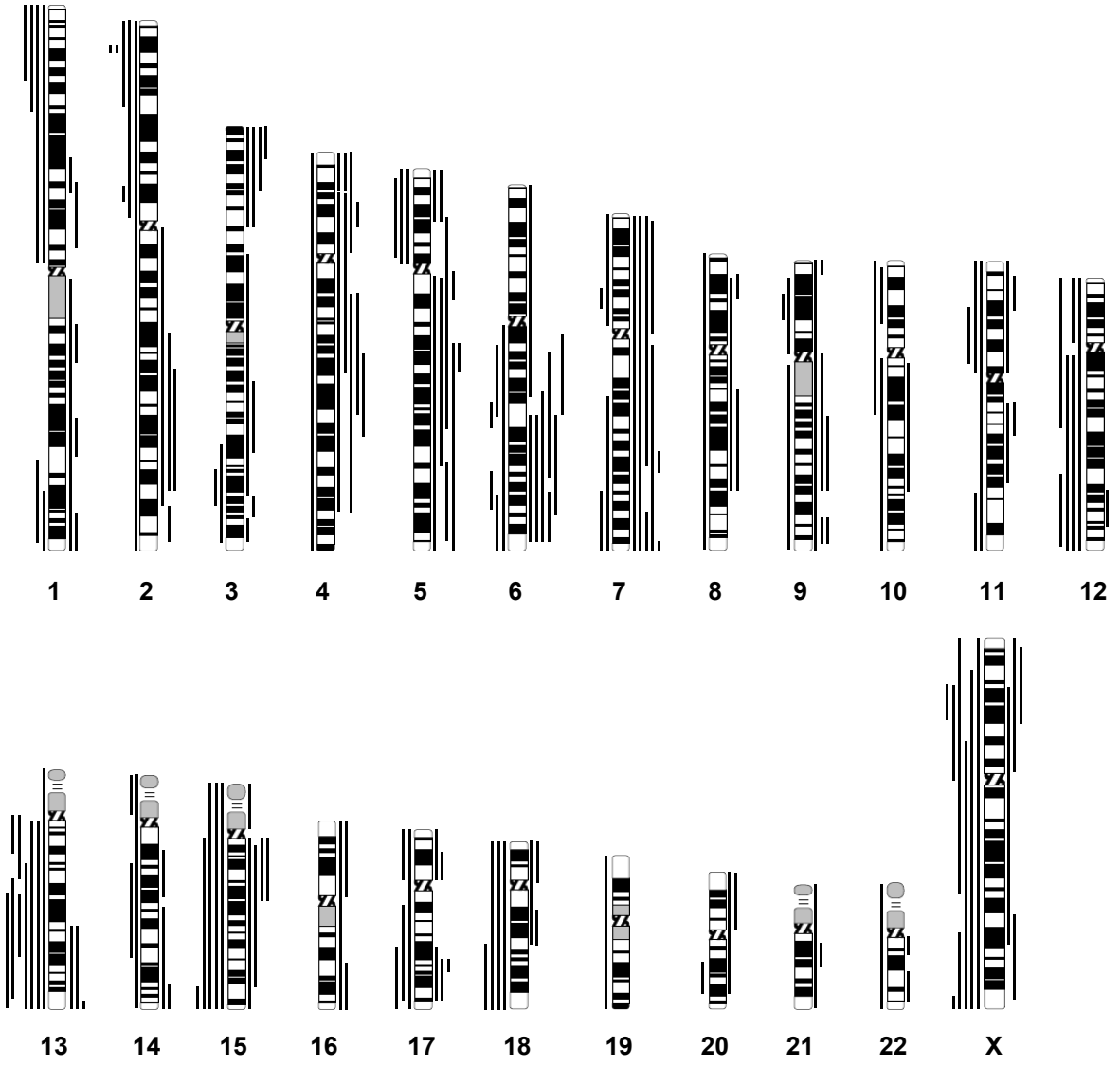

Figure 1

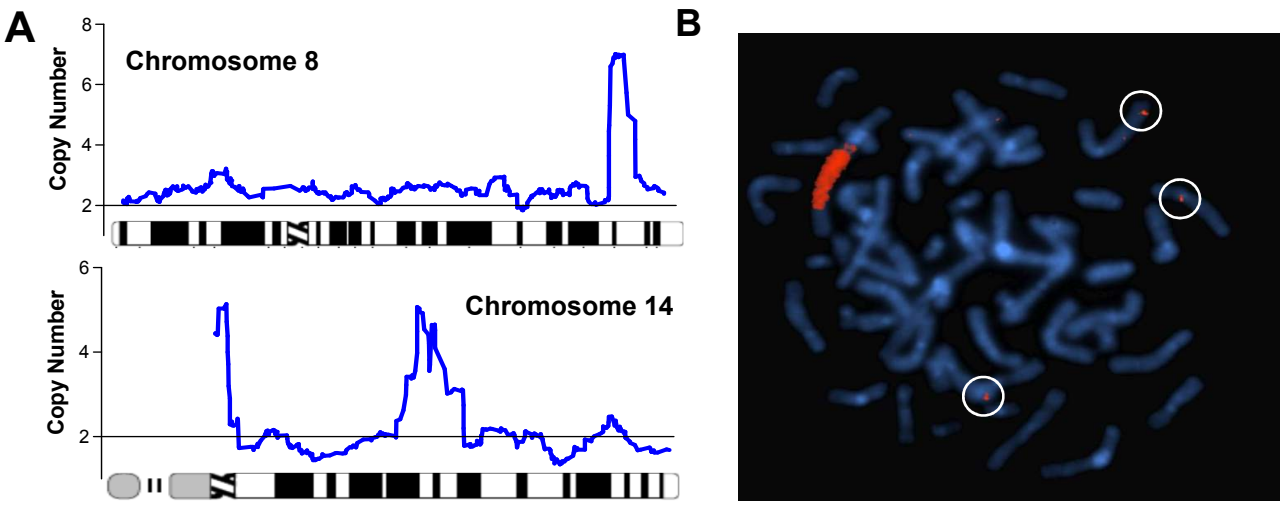
A
Chromosome 8
Copy Number
8
6
4
2
Chromosome 14
Copy Number
6
4
2
B

Figure 2

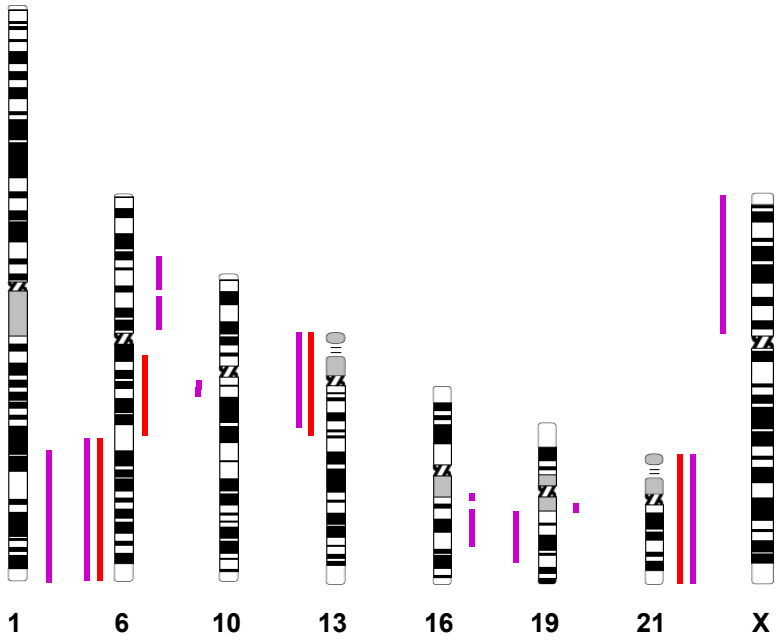

Figure 3

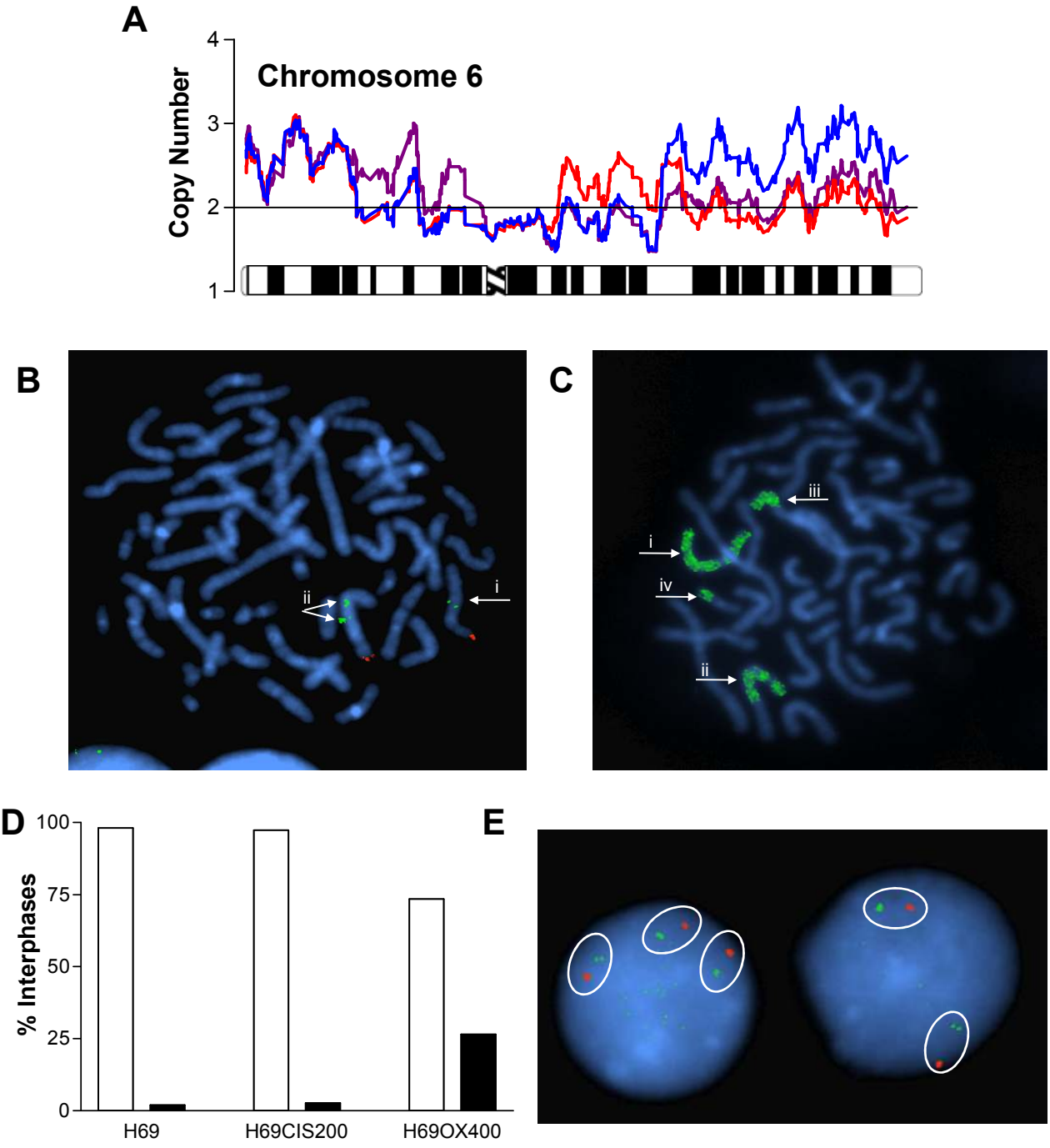
A
Chromosome 6
Copy Number
4
3
2
1
B
ii
i
C
iii
i
iv
ii
D
% Interphases
100
75
50
25
0
H69
H69CIS200
H69OX400
E

Figure 4

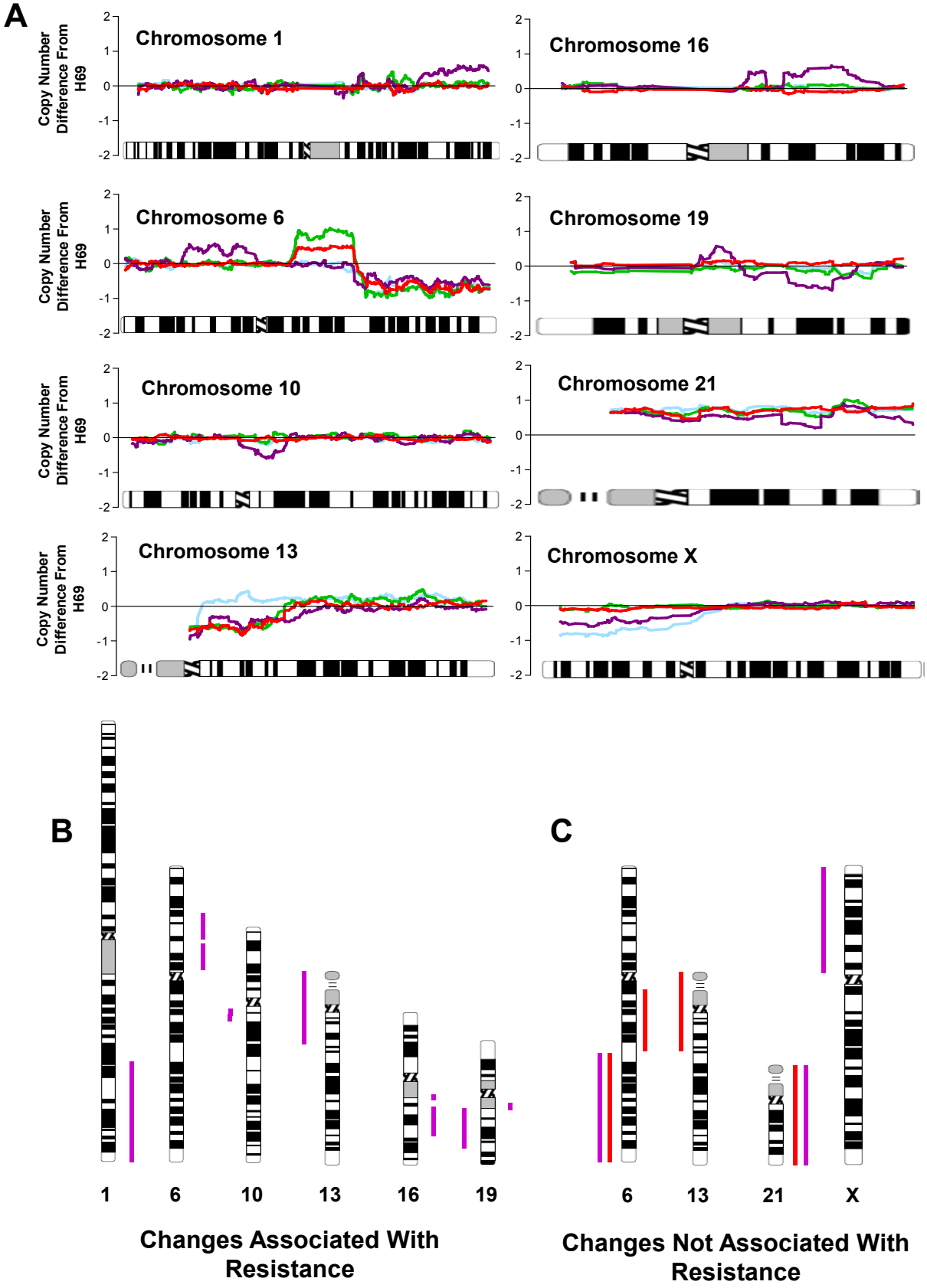
A
Copy Number Difference From H69
Chromosome 1
Chromosome 16
Chromosome 6
Chromosome 19
Chromosome 10
Chromosome 21
Chromosome 13
Chromosome X
B
1
6
10
13
16
19
Changes Associated With Resistance
C
6
13
21
X
Changes Not Associated With Resistance

Figure 5