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Advances in Brief

Comparative Genomic Hybridization Analysis of Chromosomal Alterations Induced by the Development of Resistance to Thymidylate Synthase Inhibitors[1]

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Abstract

Acquired resistance to chemotherapy is a major obstacle to the successful treatment of cancer. In the past, technical limitations prevented the detection of genetic alterations associated with such resistance on a genome-wide scale. This study evaluated comparative genomic hybridization (CGH) as a tool to detect candidate regions of the genome associated with chemoresistance. Using a variation of conventional CGH, DNA from cell lines that were resistant to thymidylate synthase inhibitors (raltitrexed and 5-fluorouracil) and their sensitive parent cells were evaluated. In MCF-7 and H630 cells that were resistant to raltitrexed, only a single region of change (18p gain) was apparent. The third cell line, H630R10, which was resistant to 5-fluorouracil, had changes in several genomic regions following the acquisition of resistance, including 18p gain. Gain in the chromosomal region containing the *thymidylate synthase* gene (18p11.32) was detected by CGH in all three resistant cell lines. However, additional novel regions of interest were identified in the cells that were resistant to 5-fluorouracil. These results suggest that CGH is of potential use in the detection of regions of the genome involved in chemoresistance.

Introduction

Acquired drug resistance is a major obstacle to the successful treatment of many tumors (1). The behavior of such tumors may be explained by the selection of a subclone of cells within the original tumor that have the ability to survive the cytotoxic effects of anticancer drugs (2). Several pathways for bypassing the effects of cytotoxic agents have been described, including: decreased influx or increased efflux across the cell membrane, compartmentalization or detoxification with intracellular lysosomes, and overproduction of the cellular target (*i.e.*, gene amplification; Ref. 3).

To date, the most common approach to the study of drug resistance has focused on the putative targets of a particular cytotoxic agent and evaluation of the biochemical pathways involved for changes at either the gene or protein level. With this approach, much has been learned about the mechanisms behind chemoresistance (4–6). However, specific study of a single gene or biochemical pathway will not detect less obvious or unrecognized genes involved in resistance.

One DNA-based technique that may be applicable to this problem is the molecular-cytogenetic technique CGH[3] (7). In a typical CGH experiment, fluorescence-labeled tumor DNA and reference DNA are competitively hybridized to donor human chromosomes. The level of signal from the fluorescent DNAs is assessed with fluorescent microscopy for each chromosome. The differences in tumor and reference signal intensity along each chromosome are a reflection of the copy number changes of corresponding sequences in the tumor DNA. CGH has the ability to globally assess the genome for regions of loss and gain in a single experiment with a limit of detection of 5–10 Mb (8). CGH can detect smaller regions of gain (~50 kb in size), if the increase in copy number is high (9).

Many new areas of alteration in the genome have been identified by CGH that had not previously been recognized to be involved in tumorigenesis (10, 11). CGH could potentially have a similar contribution to our understanding of the genetic aberrations involved in chemoresistance.

In this study, we used CGH to assess the genomes of cell lines made resistant to commonly prescribed TS inhibitors (TDX and 5-FU) as a model system to evaluate the potential of CGH as a tool to detect genomic anomalies involved in chemoresistance.

Materials and Methods

Chemicals. TDX was a kind gift of Dr. B. M. Vose at Zeneca Pharmaceuticals (Macclesfield, Cheshire, United Kingdom). 5-FU, DNA polymerase I, DNase I, Igepal CA-630, and dNTPs were obtained from Sigma Chemical Co. (London, United Kingdom). SpectrumRed dUTP and SpectrumGreen dUTP were purchased from Vysis (Surrey, United Kingdom). Human COT-1-DNA, RPMI 1640, FCS, glutamine, penicillin, and streptomycin were from Life Technologies, Inc. (Gaithersburg, MD), and formamide was from Fluka (New-Ulm, Germany). The nucleon II extraction kit was from Scotlab (Coatbridge, United Kingdom), dextran sulfate from Pharmacia Biotech (Uppsala, Sweden), and DAPI/antifade from Ampligene/Oncor (Durham, United Kingdom). All other reagents, chemicals, and buffers were purchased from British Drug Houses (Leicester, United Kingdom).

Cell Lines. Colorectal H630, H630R10, and breast MCF-7 cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units of penicillin/streptomycin in a 37°C incubator with 5% CO_2. Resistant strains were maintained in the same medium supplemented with 2 μM TDX (H630TDX and MCF-7TDX) and 10 μM 5-FU (H630R10 5-FU). All cell lines were passaged once a week using trypsin-EDTA and split 1:10, with a subsequent medium change every 3–4 days.

Comparative Genomic Hybridization. Peripheral blood lymphocytes from a healthy volunteer were cultured for 72 h at 37°C in RPMI 1640 containing phytohemagglutinin and 10% fetal bovine serum. Metaphase spreads were prepared by standard methods. Prepared slides containing metaphase spreads were viewed under phase-contrast light microscopy to select slides with a high density of nonoverlapping chromosomes. Genomic DNA from cell lines and whole blood was prepared using the nucleon II DNA extraction kit. DNA was quantified by spectrophotometry and diluted to a concentration of 0.1 μg/μl. For conventional CGH (7), control DNA (derived from a healthy individual) was labeled with SpectrumRed dUTP, and cell line DNA was labeled with SpectrumGreen dUTP. For modified CGH, the parental cell line DNA was labeled with SpectrumRed dUTP, and

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[3] The abbreviations used are: CGH, comparative genomic hybridization; TS, thymidylate synthase; TDX, ratitrexed; 5-FU, 5-fluorouracil; DAPI, 4′,6-diamidino-2-phenylindole; FR, fluorescent ratio.

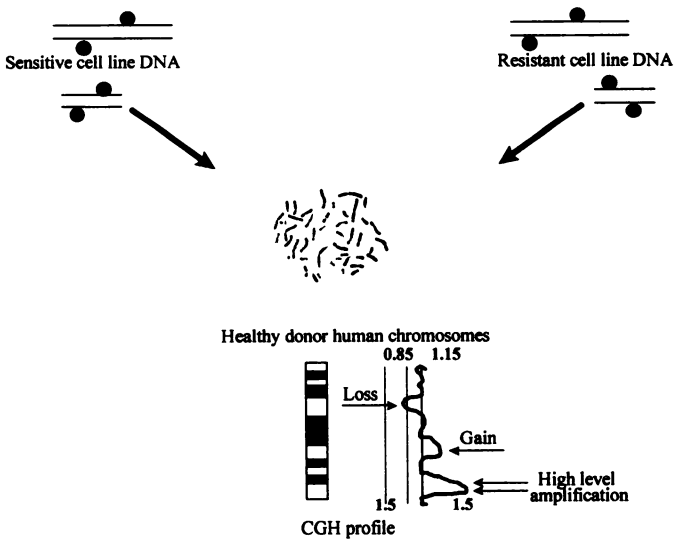

Fig. 1. Adapted CGH. Sensitive cell line DNA competes for hybridization to normal healthy donor chromosomes with the corresponding derived chemoresistant cell DNA. Thresholds for loss and gain were set at 0.85 and 1.15, respectively, and any chromosomal region with a FR of >1.5 was interpreted as high amplification.

resistant cell line DNA was labeled with SpectrumGreen dUTP (Fig. 1). All fluorochromes (SpectrumRed dUTP and SpectrumGreen dUTP) were incorporated into DNA by nick translation. Each nick translation reaction was designed to label 1 μg of genomic DNA and produce a fragment between 500 and 2000 bp. For each CGH reaction, 200 ng of labeled DNA were added to 10 μg of human COT-1 DNA. This DNA was ethanol-precipitated, dried, and dissolved in 10 μl of hybridization solution (70% formamide, 10% dextran sulfate, and 2× SSC). The DNA was then denatured at 73°C for 5 min before being added to denatured human metaphase spreads and hybridized for 3–5 days at 37°C in a humidity chamber. Before hybridization, the human metaphase spreads were denatured by exposure to 80% formamide/2× SSC at 73°C for 5 min. Posthybridization slides were washed in 0.4× SSC-0.3% Igepal CA-630 at 74°C for 3 s followed by a wash in 2× SSC-0.1% Igepal CA-630 at ambient temperature for 3 s, allowed to air dry in the dark before DAPI/antifade counterstain was added. As a negative control, 200 ng of healthy human DNA labeled with SpectrumGreen were hybridized with 200 ng of the same DNA labeled with SpectrumRed fluorochrome. To confirm equal sensitivity of both fluorochromes to detect gain and loss, we also repeated all experiments with reverse labeling of DNA. DAPI counterstain was used to karyotype all processed metaphase spreads.

Digital Image Analysis. Chromosomes in metaphase were captured using a Zeiss Axioplan II fluorescent microscope connected to a cooled, charge coupled device camera (Photometrics). Three single-color images were taken for each metaphase using a filter set specific for DAPI, SpectrumRed, and SpectrumGreen. These images were subsequently processed on a Vysis/Quips CGH software package. For each competitive hybridization reaction at least 10 metaphase spreads were analyzed. Chromosomal regions were interpreted as overrepresented (gain) if a ratio of green to red of >1.15 was observed, whereas regions with a FR of <0.85 were interpreted as underrepresented (loss). Any region with a FR of >1.5 was interpreted as high-level amplification.

Results

Detection of Resistance-specific Genome Alterations. For both of the TDX-resistant cell lines (MCF-7TDX and H630TDX), only one region of change was detected: a gain on the short arm of chromosome 18 (Fig. 2). In MCF-7TDX, the FR was 1.6, and the amplicon spanned the region from 18p11.1–11.3. An amplification of 18p11.2–11.3 was observed in H630TDX-resistant cells with a FR of 1.22. H630R10 5-FU cells also had a region of gain at 18p11.2–11.3 with a FR of 1.17. In H630R10 5-FU cells, several other novel areas of gain and loss were also detected (Fig. 3, Table 1).

Detection of Genomic Alteration in Sensitive Cell Lines. The parent cell lines were also characterized for genomic alterations (Table 2). For the breast cell line MCF-7, more regions of gain than loss were detected (14 gains/4 losses). The only region of high-level amplification in MCF-7 was on the q arm of chromosome 20 (FR = 1.95). The other two cell lines (H630 and H630R10) had several regions of gain and loss in common. For example, gains on 1q, 2p, 5p, 13q, 15q, and 16q and losses on 4q, 18q, and Y were found in both the H630 and H630R10 cell lines. However, many unique regions of loss and gain were apparent between the two cell lines (Table 2). As with the MCF-7 cell line, more regions of gain were detected than loss in H630 (16 gains/4 losses) and H630R10 (19 gains/6 losses) cells. No regions of high-level amplifications were detected in either the H630 or H630R10 cell line.

Discussion

Here, we applied a molecular cytogenetic technique, CGH, to identify regions of the genome that were associated with the acquisition of chemoresistance in human cell lines. All three cell lines that were made resistant to TS inhibitors were shown to have a gain of genetic material on the short arm of chromosome 18. The region of gain detected in each cell line spanned the region containing the *TS* gene, which has been mapped to chromosome 18p11.32 (12). Previous studies using Southern blot analysis have shown *TS* gene amplification in these cell lines after prolonged exposure to the TS inhibitors 5-FU and TDX (13, 14). These levels of *TS* gene amplification found with Southern analysis are consistent with the FRs found with adapted CGH.

The patterns of genetic aberration seen in these resistant cell lines are in accordance with our understanding of the cytotoxic mechanisms of TS inhibitors. For example, in MCF-7TDX and H630TDX cells, which were made resistant to TDX, only amplification of 18p was detected, following a genome-wide assessment. The amplified region contains the *TS* gene, consistent with its design as a specific TS inhibitor. No other mode of cytotoxicity has been described for this drug (15). This is in contrast to 5-FU, which is known to have several other modes of cytotoxicity besides TS inhibition, including incorporation into RNA (16). In addition to amplification in 18p, other novel regions of genomic alteration were detected in H630R10 5-FU-resistant cells (Table 1). The regions of loss and gain only seen in 5-FU resistance may suggest other cellular targets of this drug or a mechanism to bypass its cytotoxic effects. Database searches suggest a number of candidate genes within the regions altered in the H630R10 5-FU cells with functions capable of influencing the cytotoxic effects of 5-FU. Further cellular and molecular investigations of these regions are required before the role of these genes in resistance can be accurately assessed.

A previous study used conventional CGH to study cisplatin resistance in ovarian cell lines (17). This study did not find

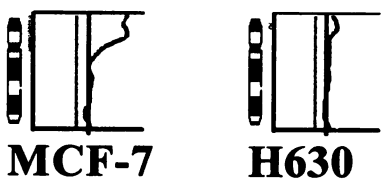

Fig. 2. Analysis of chromosome 18 by adapted CGH identified gain on 18p in DNA from the resistant MCF-7 and H630 cells.

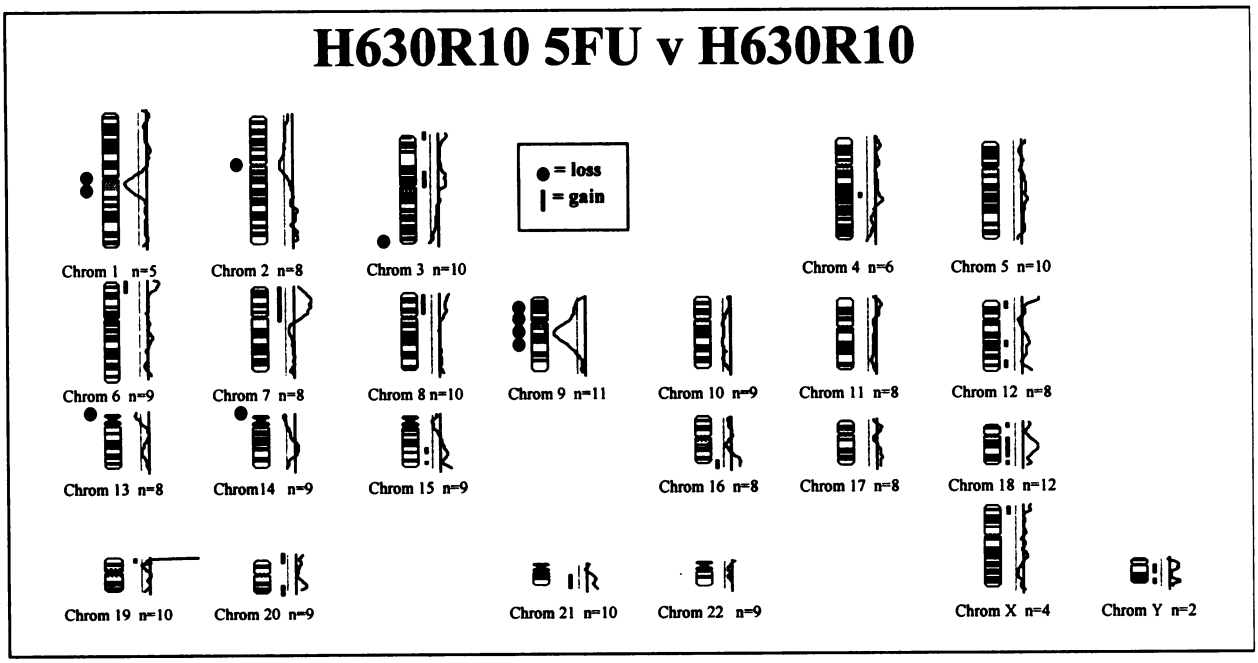

Fig. 3. CGH profile of DNA from H630R10 cells that were sensitive and resistant to 5-FU. Many areas of the genome have been detected as gained and lost, including a region of gain on 18p where the *TS* gene is mapped. *n*, number of metaphase spreads evaluated for each chromosome; *Chrom*, chromosome.

Table 1 *Regions of loss, gain, and high-level amplification (++) detected by CGH in competitive hybridizations between TS-sensitive cells and their derived resistant clones*

Cell line	TS inhibitor used	Gains	Losses
MCF-7	TDX	++18p11.1–11.3	None detected
H630	TDX	18p11.2–11.3	None detected
H630R10	5-FU	6p23–25, 7p11.1–22, 12p13.1–13.2, 15q23, 16q23–24, 18p11.2–11.3, 18q12.1–21, 20q11.2–13, Xp22.1–22.3	1q11–22, 2p11.2, 2q12, 9p22–q31

alterations in the same regions of the genome as those we found here, consistent with differences in the mechanism of action between cisplatin and TS inhibitors. That study and our own results suggest that genetic aberrations involved in chemoresistance occur across the genome and that the regions involved are specific to the type of cytotoxic agent used, although some overlap is seen between agents with shared mechanism of action (*e.g.*, TS inhibitors TDX and 5-FU both demonstrated 18p gain). Both studies suggest a role for global assessment of chemoresistance as opposed to preselection of candidate regions based on an understanding of the biochemistry of that particular anticancer agent.

Conventional CGH also detected many regions of gain and loss in the sensitive cell line DNA. Well-characterized cell lines could prove useful in identifying altered genes important in tumorigenesis. The region of high amplification detected in the MCF-7 cell line, 20q13.3, corresponds to the region where others have isolated three new genes (*AIB1*, *AIB3* and *AIB4*) commonly amplified in breast cancer (18). These investigators have found this amplification in 12–18% of primary breast tumors and 40% of breast cell lines assessed by CGH, including MCF-7 cells (19). The high degree of similarity in loss or gain between the H630 and H630R10 cell lines is not surprising because these cell lines were originally derived from the same primary cell culture. However, a number of discrepancies were noted between the cell lines, including chromosomes 2q, 3p, 4p, 6q, 7p, 10q, 12, 17, 20p, and 22q. The different CGH profiles highlight the continued genetic evolution during cell culture, which is less likely to be an accurate representation of the situation *in vivo*.

In conclusion, a common region of gain (18p) was observed in all cell lines studied with acquired resistance to TS inhibitors. This region spans that containing the *TS* gene. Using an adapted CGH protocol, we detected many novel regions of the genome that were not previously associated with chemoresistance in 5-FU-resistant cells. These regions need further study to confirm their contribution to resistance. In this study, we have shown that CGH is a technique with great potential for detecting regions of the genome involved in chemoresistance.

Table 2 *Regions of loss, gain, and high-level amplification (++) in TS-sensitive cell lines detected by conventional CGH analysis*

Cell line	Gains	Losses
MCF-7	1q22, 1q32, 3p22–24.1, 3q26.3–29, 4p15.3–16, 5p15.2, 5p15.2, 6p23–25, 7q22–31.1, 8p21–23, 8q22–24.3, 12q21.1, 14q22–24.1, 14q31.32.3, 15q21–24, 15q26, 16p11.2–13.1, 16q23–24, 17q23, 20p11.2, ++20q12–13.3, 21q22, 22q12	1p22–32, 1q12, 3q11.1, 6q11–21, −9p21, 9p24, 9q12, 10q21.1, 13q21.3–32, 13q34, 16q11.2, 17p13, Xq12
H630	1q21–23, 2p13–23, 2q11.1–13, 5p (whole arm), 7q11.23–21.1, 9p21–23, 9q21.1, 10p12–13, 11q15.5, 13q (whole arm), 15p11.2–14, 15p23–26.3, 16p12–13.12, 16q22.2, 17q21.3, 18p11.2–11.3, 20p (whole arm)	1p22–31.1, 1q11, 3p12, 4q11–31.1 5q21–22, 6q11.1, 10q26.1, 14q13, 18q21.1–23, 14q13, 18q21.3–23
H630R10	1q21, 2p15–24, 2q37, 3p24.3, 2q37, 3p24.3, 3q27–28, 4p15.2–16, 5p (whole arm), 7p15.3, 8p21–22, 9p (whole arm), 9q21.3, 10p14, 11q14, 11q23–25, 12p12, 12q24.2–24.3, 13q (whole arm), 14q32.2, 15q22–26.1, 16p12–13.2, 16q23, 17p12, 18p11.31, 19q13.2–13.3, 20q12–13.2, 22q12.3–13.2	1p22.2–31.1, 1q11.1, 3q11.1, 4q12, 5q11.2,13.2, 5q15–31.2, 14q21–28, 16q11–12, 18q11.1, 18q22.2–23

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