[CANCER RESEARCH 61, 5505–5510, July 15, 2001]

Pharmacogenomic Dissection of Resistance to Thymidylate Synthase Inhibitors[1]

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ABSTRACT

Chemoresistance is a major obstacle for successful cancer treatment. Gene amplification and altered expression are the main genetic mechanisms of tumor chemoresistance. Previously, only a limited number of genes were analyzed in each individual study using traditional molecular methods such as Northern and Southern blotting. In this study, the global gene expression patterns of 1176 genes in a panel of five thymidylate synthase (TS) inhibitor [raltitrexed (TDX) and 5-fluorouracil (5-FU)] resistant and sensitive parent cell lines were investigated using cDNA array technology. Only 28 of 1176 genes were altered >1.5-fold among resistant cells, with 2 genes (*TS* and *YES1*) consistently higher in the panel. TS mRNA and protein were consistently overexpressed in all drug-resistant tumor cell lines compared with the sensitive parent cell lines. Southern blot and FISH analysis demonstrated that the *TS* gene was amplified in 5-FU- and TDX-resistant cell lines. *YES1* mRNA and protein were overexpressed in four drug-resistant tumor cell lines but were not overexpressed in the lymphoblast cell line $W1L2_{TDX}$, although the *YES1* gene was highly amplified in these cells. The fact that W1L2 has high level (>10-fold) resistance to TS inhibitor in the absence of high *YES1* expression leads to a conclusion that *YES1* has no direct role in this drug resistance process. By narrowing the search from 1176 to 2 genes, the analysis of *in vitro* TDX and 5-FU resistance becomes more straightforward for confirmatory studies. These data provide encouragement that comprehensive transcript analysis will aid the quest for more enlightened therapeutics.

INTRODUCTION

Chemotherapy has improved the outcome of cancer treatment. However, tumor resistance to chemotherapeutic agents is still a major obstacle in the clinical management of human cancers. Numerous molecular genetic events are involved in chemoresistance to agents with distinct molecular targets. Until recently, most studies have focused on the expression of a limited number of candidate genes using traditional methods, such as reverse transcription-PCR, Northern blotting, and Western blotting. The global molecular alterations in chemoresistant tumors and cell lines are largely unknown. In contrast to traditional methods, advances in molecular technology now allow for global assessment of the genome (CGH[3]), transcriptome (cDNA microarray), or proteome (1–3). Therefore, the molecular basis for drug resistance can be evaluated without prior assumptions as to the putative mechanisms(s) of resistance. Elucidation of these genetic aberrations in drug-resistant tumor cells may help to more fully explain resistance and provide strategies to restore chemosensitivity.

To dissect the pharmacogenomics of chemoresistance, thymidylate synthase inhibitor-resistant cell lines were used as a model in this study. A critical step in the *de novo* pathway of DNA synthesis is the production of the pyrimidine nucleotide dTMP from dUMP. Because this reaction is catalyzed by TS (EC 2.1.1.45), using CH_2THF (5,10-methylene tetrahydrofolate, a folate substrate), and is the only *de novo* source of cellular thymidylate, TS is an attractive target for cytotoxic drugs (4, 5). TDX is a specific inhibitor of TS and has demonstrated activity in many solid tumors in experimental systems (6). This drug has also been used in the treatment of human tumors such as colorectal, breast, and lung cancers (7–9). Another commonly used anticancer drug, 5-FU, also inhibits TS activity, as well as damaging tumor DNA and RNA by incorporation of fluoro-nucleotides (10). As with other anticancer drugs, acquired or inherent resistance to these drugs are still the main barriers for their clinical effectiveness. The resistant cells often express high levels of TS protein, which can antagonize the cytotoxic effect of TDX and 5-FU (11). CGH results have shown that TDX-resistant cell lines have a gain of genomic material on chromosome 18 at the location of the *TS* gene (18p11.32; Ref. 12). It is possible that abnormal expression of other genes on 18p11.32 and other regions of the genome may also influence the response of tumor cells to TS inhibitors.

The aim of this study was to set up a model for the global analysis of gene expression patterns in chemoresistant cells using five pairs of TS inhibitor-sensitive and -chemoresistant tumor cell lines and to identify the common molecular genetic events involved in resistance to TS inhibitors in different tumors. Overexpression of two genes located at chromosome 18p (*TS* and *YES1*) was detected by cDNA array analysis and confirmed by Northern blotting. Gene amplification and protein overexpression status of these two genes were investigated using FISH, Southern, and Western blotting analysis. The putative relationship between gene amplification and overexpression of mRNA or protein is discussed. In addition, the data highlight the discordance between the observation of gene amplification and a true role in resistance, providing the context in which current oncogenomic studies must be interpreted.

MATERIALS AND METHODS

Cell Culture. For expression analysis, four TDX-resistant cell lines (colorectal tumor, $H630_{TDX}$ and RKO_{TDX}; breast tumor, $MCF\text{-}7_{TDX}$; lymphoblastoid, $W1L2_{TDX}$), one 5-FU-resistant cell line ($H630_{5\text{-}FU}$), and the sensitive parent cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Glasgow, United Kingdom) supplemented with 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin, and relevant concentrations of TDX ($H630_{TDX}$, 1 μM; RKO_{TDX}, $MCF\text{-}7_{TDX}$; $W1L2_{TDX}$, 2 μM) or 5-FU ($H630_{5\text{-}FU}$, 10 μM) in 75-cm^2 flasks at 37°C and 5% CO_2 until 80–90% confluent (11, 13). The cells were then harvested by trypsinization and used for further analysis.

cDNA Array Analysis. Total RNA was extracted using the method recommended by the cDNA array manufacturer (Clontech Laboratories, Palo Alto, CA). poly(A)$^+$ mRNA was extracted using the Oligotex mRNA mini purification kit (Qiagen, Inc., Santa Clarita, CA) following the supplier's instruction.

The Human Cancer 1.2 Array containing cDNAs from 1176 genes on a nylon membrane (Clontech Laboratories, Inc., Palo Alto, CA) was used to interrogate the gene expression patterns of the different cell lines. Nine housekeeping genes were also present on the membrane.[4]

Membranes were hybridized at 68°C overnight in 1.5 ml of ExpressHyb solution (Clontech) containing ^{32}P-labeled cDNA probes (>1 × 10^6 cpm/ml).

Received 2/15/01; accepted 5/8/01.

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[1] This work was supported by the Scottish Chief Scientist Office and the Aberdeen Royal Hospital Endowments.

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[3] The abbreviations used are: CGH, comparative genomic hybridization; 5-FU, 5-fluorouracil; TDX, Tomudex (raltitrexed); TS, thymidylate synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FISH, fluorescence *in situ* hybridization.

[4] The detailed information for the Human Cancer 1.2 Array is available on Clontech's web site, http://www.clontech.com/atlas/index.html.

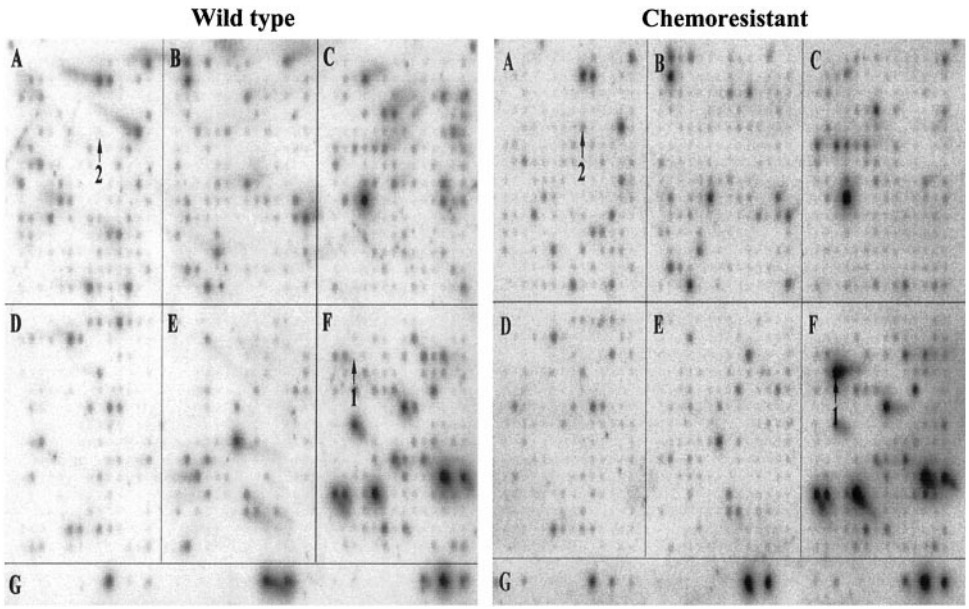

Fig. 1. The cDNA array expression pattern from MCF-7 chemoresistant and sensitive parent cells. *A–G*, categories of genes detailed by the manufacturer at www.clontech.com. *Arrows: 1, TS*; *2, YES1*.

cDNA probes were produced by reverse transcription from 1 μg of mRNA using primers specific for the 1176 genes (Clontech). The membranes were stringently washed as recommended by the supplier. The array signals were detected by exposing the membranes to a FUJI Phosphor Plate overnight. The image was scanned using a FUJI FILM-3000 PhosphorImager.

Quantitation of the cDNA Array Expression Levels. Clontech's AtlasImager software (version 1.01a) was used to analyze the signal intensity of different genes. Signals expressed at >1.5-fold higher or 0.5-fold lower than the external background signal were analyzed further. Intensities of expression from each membrane were normalized using ubiquitin, because it had the smallest interassay variation of the nine housekeeping genes on the membrane (data not shown). The expression patterns of the paired cell lines were compared using the AtlasImager software. Variation in gene expression between duplicate experiments was <20% for 95% of the genes on the array (data not shown). To eliminate false-positives, only genes expressed at least 1.5-fold higher or <0.5-fold lower in the resistant cells compared with the parent sensitive cells were interpreted as over- or underexpressed, respectively.

Northern Blot Analysis. Consistent alteration of gene expression detected on the cDNA arrays in different cell lines was confirmed by Northern hybridization. Twenty μg of total RNA were electrophoresed through a 1% agarose gel containing 1.9% formaldehyde, and size-fractionated RNA was transferred to a Hybond-N^+ nylon membrane (Amersham, Buckinghamshire, United Kingdom) by capillary transfer overnight. The blotted RNA was immobilized by exposure to 250 nm UV light for 3 min. Membranes were hybridized with [^{32}P]dATP random labeled probes at 68°C overnight. The membranes were exposed to Kodak X-ray film overnight at −70°C. The intensities of the bands were analyzed using Bio-Rad Molecular Analyst software. Blots were stripped and rehybridized with different probes. *TS* and *YES1* genes were subjected to Northern blotting analysis, and the *GAPDH* gene was used as an internal control. The probes were reverse transcribed and amplified from 1 μg of

Table 1 *The genes with altered expression in chemoresistant cell lines compared with the sensitive parent cell lines (resistant/sensitive ratio)*

Genes with a ratio >1.5 or <0.5 are shown in bold. The numbers in the table represent the gene expression ratio between chemoresistant and chemosensitive cell lines. 1.5 and 0.5 are set as threshold of over- and underexpression, respectively.

Gene	W1L2-TDX	RKO-TDX	MCF-TDX	H630-TDX	H630-5FU
Thymidylate synthase	**3.61**	**3.54**	**5.80**	1.45	**2.53**
YES1 proto-oncogene	**0.41**	**3.27**	**3.23**	**1.82**	**2.49**
T-cell protein-tyrosine phosphatase	0.53	**4.19**	**3.84**	1.21	1.15
Activator 1 40-kDa subunit	0.88	1.22	**2.10**	NE[a]	0.75
CAK1 antigen	NE	0.79	**1.53**	0.78	**8.53**
RPD3 protein	0.56	0.86	1.38	NE	**2.01**
Checkpoint suppressor 1	1.10	0.89	1.24	1.07	**0.35**
α1 catenin	0.97	1.06	1.22	NE	**3.18**
TGF-β3	0.79	0.84	1.11	NE	**2.03**
Semaphorin III	0.88	0.79	1.09	NE	**2.82**
Ephrin type-B receptor 2 precursor	1.03	1.44	1.09	0.93	**2.28**
Lymphotoxin β receptor precursor	0.61	0.66	1.08	NE	**2.02**
c-*rel* proto-oncogene protein	0.95	1.21	1.02	1.36	**0.36**
Stem cell factor precursor	NE	NE	0.99	1.08	**5.28**
Interleukin-4 precursor	NE	1.22	0.95	0.68	**2.33**
Interferon-inducible protein 9–27	0.89	0.89	0.92	1.23	**0.26**
CD91 antigen	1.12	0.94	0.91	NE	**0.46**
Fibroblast growth factor receptor 1 precursor	1.09	1.21	0.67	0.88	**6.18**
p68-trk-T3 oncoprotein	NE	NE	0.63	1.32	**0.11**
Procollagen 3 α1 subunit precursor	**1.61**	0.73	0.62	0.97	**4.52**
Integrin α9	1.40	0.81	0.59	1.13	**2.32**
β NGF	1.31	**1.55**	0.59	0.99	**0.38**
Retinoic acid receptor α1	NE	**2.92**	**0.40**	1.42	0.69
PRSM1 metallopeptidase	0.72	1.12	**0.40**	NE	1.18
Mitotic feedback control protein MADP2 homologue	0.97	1.14	**0.40**	NE	0.88
c-*myc* oncogene	1.26	1.20	**0.29**	1.32	0.89
Metalloproteinase inhibitor 3 precursor	1.26	1.28	NE	NE	**2.30**
DNA topoisomerase IIβ	0.88	NE	NE	0.97	**0.46**

[a] NE, not evaluable.

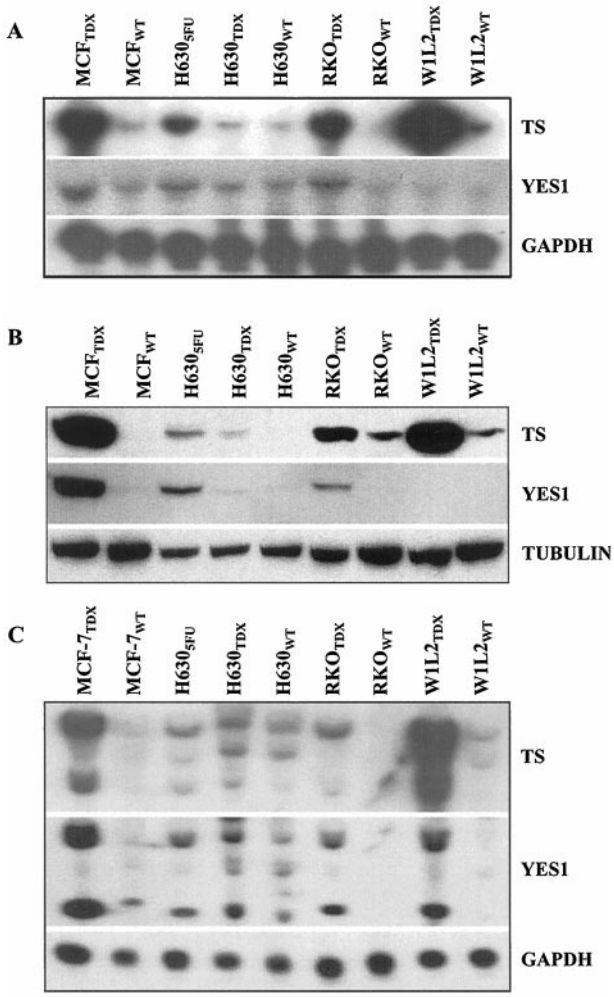

Fig. 2. Expression of *TS* and *YES1* mRNA and protein and gene copy number. *A*, Northern blotting analysis: expression levels of TS and YES1 mRNA in 20 μg of total RNA, detected using specific radiolabeled cDNA probes. GAPDH was used as an internal standard control. *B*, Western blotting analysis: protein levels of TS and YES1 in 200 μg of total protein, detected using relevant antibodies (see "Materials and Methods"). Tubulin was used as an internal standard control. *C*, Southern blotting analysis: genomic DNA (20 μg/lane) was cut with *Hin*dIII and hybridized with the same cDNA probes used for Northern blotting analysis. GAPDH was used as standard control.

MCF-7$_{TDX}$ total RNA using a one-step reverse transcription-PCR kit (Promega, Southampton, United Kingdom). The primer sequences are as follows: for *TS* (1055 bp), forward 5′-AGCCTGAGAGATGAATTCCCTC-3′ and reverse 5′-CGTGGGATTGAAATGCACATAC-3′; for *YES1* (857 bp), forward 5′-CAGAACCCACTACAGTGTCACC-3′ and reverse 5′-TTGAAGGAAAGCTTCTGGCATC-3′; and for *GAPDH* (915 bp), forward 5′-AAGGTCGGAGTCAACGGATTTG-3′ and reverse 5′-CTTGACAAAGTGGTCGTTGAGG-3′. The sequence of each amplified probe was confirmed by direct automated DNA sequencing.

Southern Blot Analysis. The DNA was extracted using a Nucleon DNA purification kit (Nucleon Biosciences, Didsbury, United Kingdom) following the supplier's instruction. Twenty μg of DNA from each cell line were digested with *Hin*dIII for 3 h at 37°C. The digested DNA fragments were separated through a 0.6% agarose gel. After being denatured in 0.1 N NaOH, the DNA was blotted onto a nylon membrane by overnight capillary transfer and fixed under 250 nm UV light for 3 min. The probes and hybridization conditions were the same as those for Northern blotting analysis. The same membrane was stripped and hybridized to different probes.

Fluorescence *in Situ* Hybridization. A P1 clone containing the *TS* gene was obtained from Genome System, Inc. (St. Louis, MO) using primers located in exon 5 of the *TS* gene (primers, 5′-CTTCCTCTGATGGCGCTG-3′ and 5′-CCGTGATGTGCGCAATC-3′). P1 probe labeling, and hybridization onto metaphases from human cancer cell lines was carried out as described previously (14). Slides were analyzed using an Axioplan II microscope (Zeiss, Welwyn Garden City, United Kingdom). Images were captured using a Sensys CCD camera (Photometrics, Tucson, AZ) with Quips software (Vysis, Richmond, Surrey, United Kingdom).

Western Blot Analysis. Cell lines were washed in ice-cold PBS and lysed in 500 μl of RIPA buffer. The lysate was centrifuged for 5 min in a microcentrifuge, and the supernatants were retained. The protein (200 μg/cell line) was electrophoresed through a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Hertfordshire, United Kingdom) using an electrophoretic transfer chamber (Millipore). The blots were blocked for nonspecific binding by incubating the membranes for 1 h in TBS-T with 5% nonfat milk, which was also used to dilute primary (*TS*, LabVision, Fremont, CA; 1:250; *YES1*, Wako, Osaka, Japan 1:200) and secondary (Amersham; 1:5000) antibodies. The signal was detected using an ECL Western blotting detection kit (Amersham) and visualized by exposure to X-ray film. Reprobe of the membrane with an anti-tubulin monoclonal antibody (Sigma Chemical Co.) was used to standardize the samples.

RESULTS

Array Analysis of Gene Expression in Chemoresistant Cell Lines. The expression patterns of cell lines resistant to TS inhibitors were compared with those of the wild-type cell lines (Fig. 1). A total of 28 of 1176 genes (2.4%) had altered expression in the different chemoresistant cell lines (Table 1). More genes were altered in H630$_{5\text{-FU}}$ cells (22 genes) than the TDX-resistant cell lines (1–9 genes; Table 1). However, only *TS* and *YES1* genes were consistently overexpressed, with a resistance:sensitive ratio $\geq$1.5 in 5 (*TS*) and 4 (*YES1*) of the chemoresistant cell lines. *YES1* mRNA was not overexpressed in W1L2$_{TDX}$ cells in which TS mRNA was markedly overexpressed (Table 1). Because *TS* and *YES1* are within 50 kb of

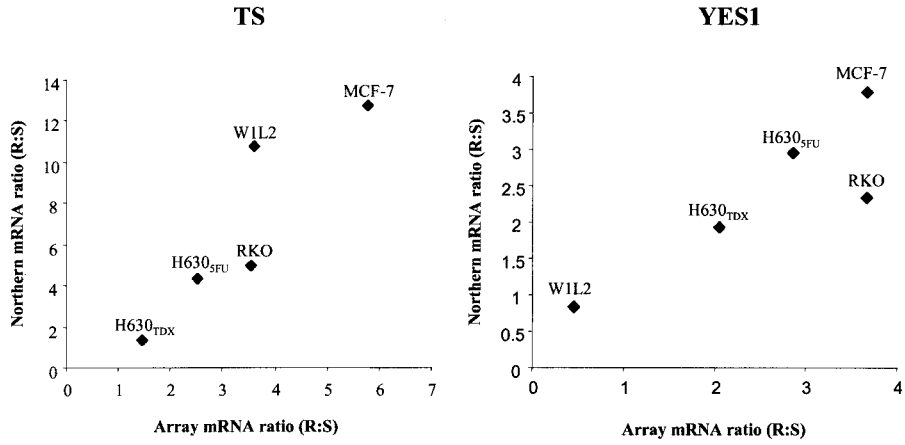

Fig. 3. Comparison of *TS* and *YES1* mRNA expression levels detected by cDNA array or Northern blotting analysis, expressed as the ratio of resistant:sensitive cell lines (*R:S*). The Northern blot analysis of both genes was standardized using GAPDH.

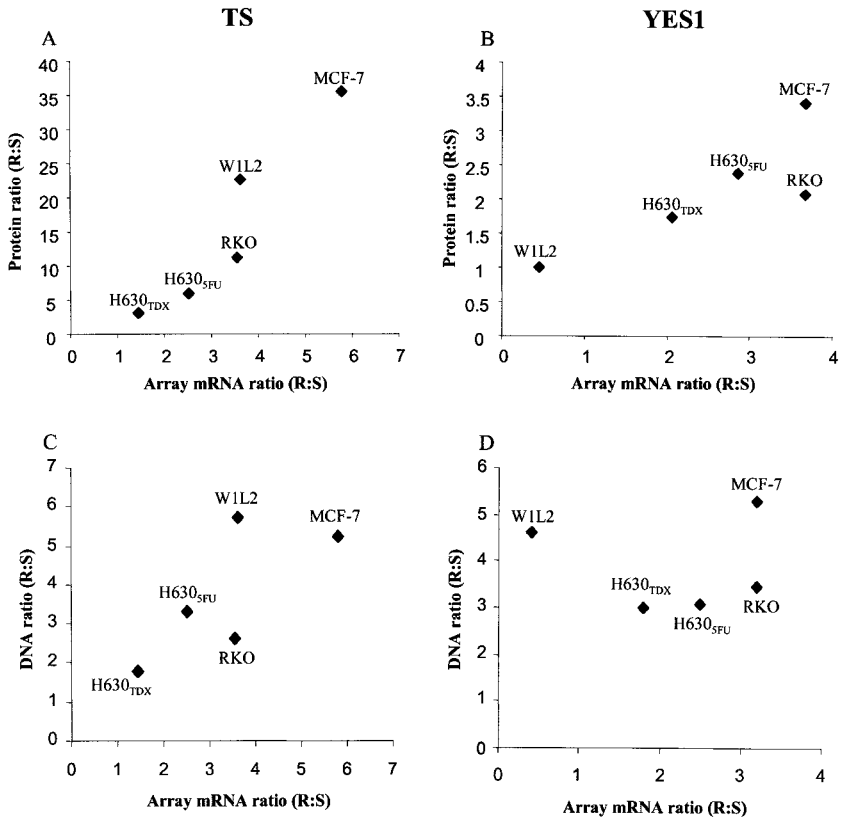

Fig. 4. Relationship between the ratio of *TS* (*A* and *C*) or *YES1* (*B* and *D*) mRNA in resistant:sensitive cells and TS:YES1 protein expression ratio (*A* and *B*) or DNA copy ratio (*C* and *D*) in the five cell lines.

each other at chromosome 18p11.32 (15), further genomic analysis was performed to dissect the involvement of the two genes in resistance.

Comparison of *TS* and *YES1* DNA, mRNA, and Protein. To confirm the cDNA array results, the expression levels of the *TS* and *YES1* genes were determined by Northern blot analysis (Fig. 2*A*). The relative expression levels of *TS* and *YES1* were closely matched between cDNA array and Northern blotting (Fig. 3). The expression of TS and YES1 protein was analyzed by Western blotting (Fig. 2*B*). The protein expression levels in each cell line reflected mRNA expression, with corresponding increase in TS and YES1 within four of five cell lines (Fig. 4, *A* and *B*). W1L2 did not demonstrate a significant change in YES1 protein between sensitive and resistant cells (Figs. 2*B* and 4*B*).

Gene amplification is one of the most common mechanisms of overexpression in cancer cells, and Southern analysis demonstrated that the *TS* and *YES1* genes were amplified in all chemoresistant cell lines (Figs. 2*C* and 4, *C* and *D*). TS gene amplification ranged from 1.8- to 5.7-fold, whereas *YES1* was amplified 3.0–5.3-fold in the resistant cells. In each chemoresistant cell line, the *TS* and *YES1* genes were amplified at a comparable level (Fig. 5). FISH was performed using a P1 clone containing TS to confirm gene amplification at the cytogenetic level (Fig. 6). In concordance with the Southern analysis, MCF-7$_{TDX}$, RKO$_{TDX}$, and W1L2$_{TDX}$ show the largest degree of TS amplification by FISH (>20 copies), compared with H630$_{5FU}$ (>10 copies) and H630$_{TDX}$ (6 copies).

The relative *TS* and *YES1* mRNA expression levels in chemosensitive cell lines were evaluated to provide context to the findings in the resistant cells. W1L2 wild-type cells expressed much lower *YES1* mRNA, compared with the other wild-type cell lines (1.9–2.8-fold lower). In contrast, TS mRNA was 1.3–2.0-fold higher in W1L2 wild-type compared with the other wild-type cell lines.

DISCUSSION

Chemoresistance is a major barrier for the successful treatment of malignant diseases with chemotherapy. The global analysis of gene expression patterns in drug-resistant cell lines may help to more fully elucidate the underlying mechanisms involved. In this study, the gene expression profiles of five TS inhibitor-resistant cell lines were compared with parent cell lines using a cDNA array. Surprisingly, only 2 of 1176 genes (0.17%) were consistently altered across the cell line panel, *TS* and *YES1*. Overexpression of TS is strongly associated with the resistance of cell lines to TS inhibitors (11, 16). cDNA array analysis showed high expression of TS mRNA in all TDX- and 5-FU-resistant cell lines (Fig. 1). Overexpression of *YES1* mRNA was also observed in four of five drug-resistant cell lines. The cDNA array results were confirmed by Northern blot analysis. Western analysis

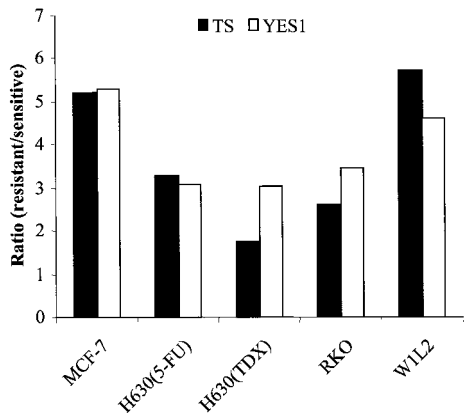

Fig. 5. Comparison of the ratio of *TS* or *YES1* DNA copy number in paired chemoresistant:chemosensitive cell lines.

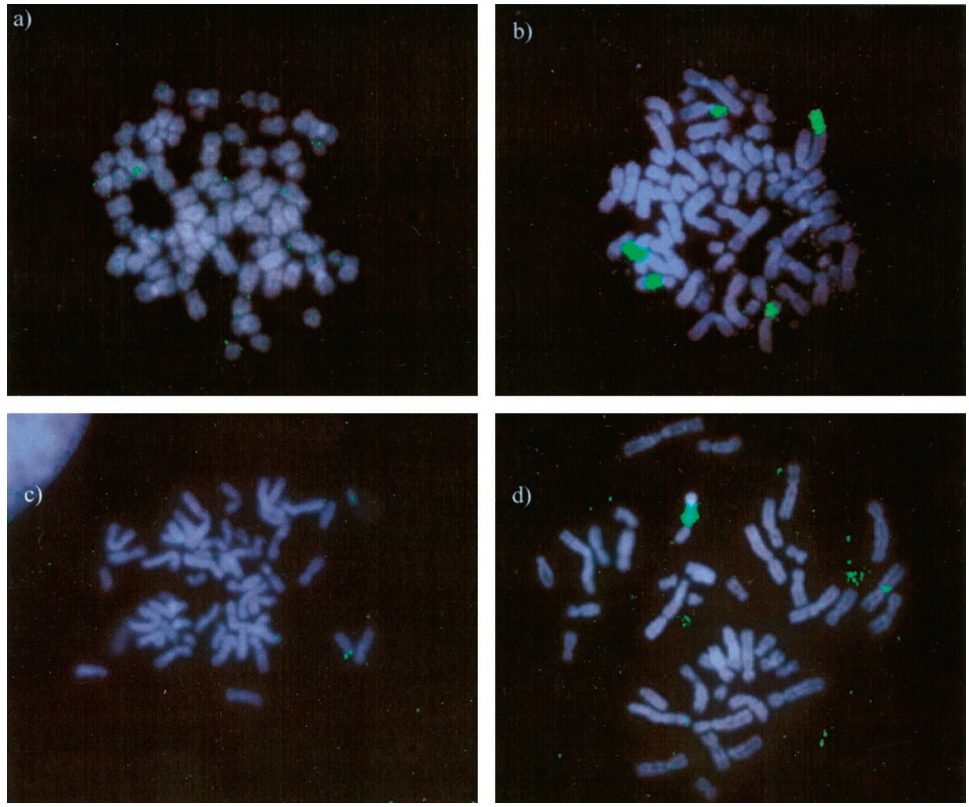

Fig. 6. FISH with a TS-containing P1 probe showing amplification of the TS region of 18p in resistant cells. *a*, MCF-7 sensitive; *b*, MCF-7_{TDX}; *c*, WIL2 sensitive; *d*, $WIL2_{TDX}$.

showed that TS and YES1 protein levels had the same pattern of expression as that observed for mRNA in the drug-resistant cell lines. DNA analysis found concordance between *TS* and *YES1* gene amplification. These results are not unexpected, because the *TS* and *YES1* genes lie 50 kb apart on chromosome 18p11.32 (15). Previous CGH studies showed an amplification of genomic material at 18p11.32 and beyond in the resistant cell lines (12), and FISH data demonstrated a large amplicon of the TS-containing region of 18p in drug-resistant cell lines (Fig. 6). Therefore, it is likely that the amplicon would contain both *TS* and *YES1*. However, the discordance between DNA-mRNA-protein for the resistant:sensitive cells is surprising. No alteration in *YES1* mRNA was observed in the $W1L2_{TDX}$ cell line in the context of *YES1* gene amplification and *TS* overabundance at the DNA, mRNA, and protein level.

These findings have important implications for the interpretation of data in the postgenome era. There have been a large number of putative oncogenes and tumor suppressor genes that are the "victims" of guilt by association or, more precisely, localization. For example, over 10 different genes in chromosome 20q have been found to be amplified in a number of human solid tumors and purported to have defining roles in tumor biology (14, 17). However, CGH analysis has found the entire 20q arm to be amplified, suggesting that many amplified genes are being "taken along for the ride" rather than "driving" the malignant process (14). The data presented here support this theory. Although the *TS* and *YES1* genes are both amplified in all of the resistant cells, only TS is strongly overexpressed at the mRNA and protein levels in all cell lines. *YES1* is an oncogene with kinase activity in a number of solid tumors, including colon (18, 19). W1L2 is a lymphoblastoid cell line, whereas the others are derived from carcinomas of colon and breast, consistent with tissue-specific transcriptional control as the basis for the discordance between gene copy and mRNA/protein in this cell line. The fact that W1L2 has high level (>10-fold) resistance to TS inhibitor in the absence of high *YES1* expression leads to a conclusion that *YES1* has no direct role in this drug resistance process.

The FISH analysis showed that TS amplification occurs as a highly replicated amplicon, rather than as a double minute chromosome or random integration into the genome. Therefore, strategies to reverse resistance from TS amplification will need to use direct approaches [*e.g.*, TS ribozymes (20) rather than chemoelimination of extra chromosomal DNA (21)].

A major concern with the use of cDNA microarray analysis in cancer therapeutics is that the assessment of a large number of genes (*i.e.*, 1176 in the Atlas array) may identify an overwhelming number of "associated" genes. This would lead to a great amount of fruitless effort to try and confirm involvement in resistance and bring confusion, rather than clarity, to the understanding of drug action. However, this study is an example of the promise of the microarray approach. Although multiple genes ($n = 28$) were altered in the various resistant clones, only *TS* and *YES1* were changed consistently. By narrowing the search from 1176 to 2 genes, the analysis of *in vitro* TDX and 5-FU resistance becomes more straightforward for confirmatory studies. Similar approaches can now be conducted in clinical samples to establish the *in vivo* mechanisms of resistance to TDX, 5-FU, and other active agents. It is not known if a proportional degree of clarity would be reached with microarrays containing larger numbers of genes. These data provide encouragement that comprehensive transcript analysis will aid the quest for more enlightened therapeutics.

ACKNOWLEDGMENTS

Many thanks to Professor Patrick Johnston and Professor Ann Jackman for the sensitive and resistant cell lines and to Dr. Vose at Zeneca, United Kingdom for Tomudex.

REFERENCES

1. Rooney, P. H., Murray, G. I., Stevenson, D. A., Haites, N. E., Cassidy, J., and McLeod, H. L. Comparative genomic hybridization and chromosomal instability in solid tumours. Br. J. Cancer, *80:* 862–873, 1999.
2. Scherf, U., Ross, D. T., Waltham, M., Smith, L. H., Lee, J. K., Tanabe, L., Kohn, K. W., Reinhold, W. C., Myers, T. G., Andrews, D. T., Scudiero, D. A., Eisen, M. B., Sausville, E. A., and Pommier, Y., Botstein, D., Brown, P. O., and Weinstein, J. N. A gene expression database for the molecular pharmacology of cancer. Nat. Genet., *24:* 236–244, 2000.
3. Chambers, G., Lawrie, L., Cash, P., and Murray, G. I. Proteomics: a new approach to the study of disease. J. Pathol., *192:* 280–288, 2000.
4. Jackman, A. L., and Calvert, A. H. Folate-based thymidylate synthase inhibitors as anticancer drugs. Ann. Oncol., *6:* 871–881, 1995.
5. Rustum, Y. M., Harstrick, A., Cao, S., Vanhoefer, U., Yin, M-B., Wilde, H., and Seeber, S. Thymidylate synthase inhibitors in cancer therapy: direct and indirect inhibitors. J. Clin. Oncol., *15:* 389–400, 1997.
6. Touroutoglou, N., and Pazdur, R. Thymidylate synthase inhibitors. Clin. Cancer Res., *2:* 227–243, 1996.
7. Van Cutsem, E. Raltitrexed (Tomudex) in combination treatment for colorectal cancer. Eur. J. Cancer, *35:* S1–S2, 1999.
8. Judson, I. R. Tomudex (raltitrexed) development: preclinical, Phase I and II studies. Anticancer Drugs, *8:* S2, S5–S9, 1997.
9. Vokes, E. E., Goh, B. C., Bertucci, D., Vogelzang, N. J., Mani, S., and Ratain, M. J. A Phase I study of raltitrexed and paclitaxel given every 3 weeks to patients with solid tumors. Cancer (Phila.), *86:* 528–532, 1999.
10. Pinedo, H. M., and Peters, G. F. J. Fluorouracil. Biochemistry and pharmacology. J. Clin. Oncol., *6:* 1653–1664, 1986.
11. Jackman, A. L., Kelland, L. R., Kimbell, R., Brown, M., Gibson, W., Ahene, G. W., Hardcastle, A., and Boyle, F. T. Mechanisms of acquired resistance to the quinazoline thymidylate synthase inhibitor ZD1694 (Tomudex) in one mouse and three human cell lines. Br. J. Cancer, *71:* 914–924, 1995.
12. Rooney, P. H., Stevenson, D. A. J., Marsh, S., Johnston, P. G., Haites, N. E., Cassidy, J., and McLeod, H. L. Comparative genomic hybridization analysis of chromosomal alterations induced by the development of resistance to thymidylate synthase inhibitors. Cancer Res., *58:* 5042–5045, 1998.
13. Johnston, P. G., Behan, K. A., Allegra, C. J., Drake, J. C. Fluorouracil: active in ZD1694 (Tomudex) resistant cell lines with markedly elevated thymidylate synthase levels. J. Natl. Cancer Inst., *87:* 1558–1559, 1995.
14. Boonsong, A., Marsh, S., Rooney, P. H., Stevenson, D. A. J., Cassidy, J., and McLeod, H. L. Characterization of the topoisomerase I locus in human colorectal cancer. Cancer Genet. Cytogenet., *121:* 56–60, 2000.
15. Silverman, G. A., Kou, W. L., Taillon-Miller, P., and Gray, J. W. Chromosomal reassignment: YACs containing both YES1 and thymidylate synthase map to the short arm of chromosome 18. Genomics, *15:* 442–445, 1993.
16. Copur, S., Aiba, K., Drake, J. C., Allegra, C. J., and Chu, E. Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. Biochem. Pharmacol., *49:* 1419–1426, 1995.
17. Collins, C., Rommens, J. M., Kowbel, D., Godrey, T., Tanner, M., Hwang, S., Polikoff, D., Nonet, G., Cochran, J., Myambo, K., Jay, K. E., Froula, J., Cloutier, T., Kuo, W., Yaswen, P., Dairkee, S., Giovanola, J., Hutchinson, G. B., Isola, J., Kallioniemi, O., Palazzolo, M., Martin, J., Ericsson, C., Pinkel, D., Albertson, D., Li, W., and Gray, J. W. Positional cloning of *ZNF217* and *NABC1*: genes amplified at 20q13.2 and overexpressed in breast carcinoma. Proc. Natl. Acad. Sci. USA, *95:* 8703–8708, 1998.
18. Park, J., Meisler, A. I., and Cartwright, C. A. C-Yes tyrosine kinase activity in human colon carcinoma. Oncogene, *8:* 2627–2635, 1993.
19. Han, N. M., Curley, S. A., and Gallick, G. E. Differential activation of pp60c-*src* and pp62c-Yes in human colorectal carcinoma liver metastases. Clin. Cancer Res., *2:* 1397–1404, 1996.
20. Kobayashi, H., Takemura, Y., Miyachi, H., Skelton, L., and Jackman, A. L. Effect of hammerhead ribozyme against human thymidylate synthase on the cytotoxicity of thymidylate synthase inhibitors. Jpn. J. Cancer Res., *86:* 1014–1018, 1995.
21. Von Hoff, D. D., Waddelow, T., Forseth, B., Davidson, K., Scott, J., and Wahl, G. Hydroxyurea accelerates loss of extrachromosomally amplified genes from tumor cells. Cancer Res., *51:* 7263–7269, 1991.

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Cancer Res 2001;61:5505-5510.