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Mechanistic and Predictive Profiling of 5-Fluorouracil Resistance in Human Cancer Cells

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ABSTRACT

Gene expression was analyzed in five pairs of 5-fluorouracil (5-FU) resistant and parental cancer cell lines on DNA microarrays. In unsupervised analysis, a prediction rule was built from the expression profiles of 29 genes, and 5-FU sensitivity class was predicted with 100% accuracy and high predictive strength. In supervised analysis of key 5-FU pathways, expression of 91 genes was associated with 5-FU sensitivity phenotype and segregated samples accordingly in hierarchical analysis. Key genes involved in 5-FU activation were significantly down-regulated (thymidine kinase, 2.9-fold; orotate phosphoribosyltransferase, 2.3-fold; uridine monophosphate kinase, 3.2-fold; pyrimidine nucleoside phosphorylase 3.6-fold) in resistant cells. Overexpression of thymidylate synthase and its adjacent gene, c-Yes, was detected in the resistant cell lines. The mRNA and protein overexpression of nuclear factor κB (NFκB) p65 and related antiapoptotic c-Flip gene was detected in resistant cells. The 5-FU–resistant cell lines also showed high NFκB DNA-binding activity. Cotransfection of NFκB p50 and p65 cDNA induced 5-FU resistance in MCF-7 cells. Both NFκB- and 5-FU–induced resistant cell lines manifested reduced expression of genes governing G_1-S and S-phase transition. Expression of genes involved in DNA replication was also down-regulated in resistant cell lines. These findings were highly consistent with the slower growth rate, higher proportion of G_1, and lower proportion of S-phase cells in the resistant cell lines. This phenotype may protect resistant cells from cell death induced by incorporation of 5-FU into DNA chains, by allowing time to repair 5-FU–induced damage. Our findings may provide novel targets for tackling 5-FU resistance.

INTRODUCTION

5-Fluorouracil (5-FU) is a widely used chemotherapeutic agent that inhibits cancer cell growth and initiates apoptosis by targeting thymidylate synthase (TS) and by direct incorporation of 5-FU metabolites into DNA and RNA. 5-FU–based chemotherapy improves overall and disease-free survival of patients with colorectal, breast, head and neck and aerodigestive cancers. The combination of 5-FU with other newly developed anticancer drugs such as irinotecan, Tomudex (TDX), and oxaliplatin has improved response rates for advanced colorectal cancer (CRC) from 40 to 50% (1). Despite these improvements, there are <12% of advanced CRC patients, who have received systemic 5-FU chemotherapy, still alive after 2 years (2, 3). *De novo* and acquired chemoresistance is the major obstacle for the success of 5-FU–based chemotherapy. Although TS protein overexpression is a major 5-FU resistance-inducing factor (1, 4, 5), TDX-resistant cells with high TS levels are only moderately resistant to 5-FU (RKO_{TDX}, $H630_{TDX}$, and $W1L2_{TDX}$, 2- to 3-fold increased IC_{50} compared with the sensitive parental lines[3]). High TS expression does not account for all nonresponding tumors in colorectal cancer patients treated with 5-FU (2). 5-FU sensitivity is also influenced by expression levels of dihydropyrimidine dehydrogenase, the genetic status of p53, and DNA mismatch-repair genes (1). The experimental and clinical data about the predictive value of the above factors are still quite controversial. In addition, the precise molecular mechanisms of 5-FU chemoresistance in cancer patients are still largely unknown. Therefore, identification and evaluation of new molecular targets involved in 5-FU resistance will allow improved scheduling regimes and development of novel agents, to improve the tumor response to cancer chemotherapy.

The sensitivity of cancer cells to 5-FU is influenced by multiple molecular events. Global analysis of the molecular alterations in 5-FU–resistant cancer cells is required to unravel the complex mechanisms of 5-FU chemoresistance. Microarray technology, developed in recent years, has enabled analysis of pan-genomic expression profiles in cells or tissues of interest (6). When DNA microarray technology is used, complex pathologic events can be intricately probed. A combination of microarray and traditional molecular technologies will enable us to functionally characterize genes related to anticancer drug resistance and identify novel molecular targets for anticancer drug development.

Using Affymetrix HG-U133A oligonucleotide microarrays consisting of 22,283 transcripts representing approximately 16,000-classified human genes, we have analyzed the expression profiles of five pairs of 5-FU–resistant and relevant drug sensitive parental cancer cell lines. Specific molecular factors and cellular pathways mediating and/or predictive of 5-FU resistance were elucidated in this study.

MATERIALS AND METHODS

Cell Lines and Materials. Cell Culture and Cytotoxicity Analysis. The 5-FU–resistant colorectal cancer cell line H630-R10 and the parental cell line $H630_{WT}$ were generously provided by Prof. P. G. Johnston (Department of Oncology, The Queen's University of Belfast). The subclones ($H630B_{WT}$ and H630B-R10), derived by prolonged independent culture (>5 years) of the original $H630_{WT}$ and H630-R10 cell lines, respectively, were generated to evaluate acquired changes in the 5-FU–resistant phenotype. The 5-FU–resistant breast cancer cell lines ($MCF\text{-}7_{FU1}$, $MCF\text{-}7_{FU5}$, and $T47D_{FU2.5}$) were generated by continuously culturing the drug-sensitive parental cell lines ($MCF\text{-}7_{WT}$ and $T47D_{WT}$) in medium containing increasing concentrations of 5-FU in a stepwise procedure over 2 years. All cell lines were cultured in RPMI 1640 (Invitrogen, Paisley, United Kingdom) supplemented with 10% FCS, 50 units/mL penicillin, 50 μg/mL streptomycin. Resistant cell lines were maintained in the presence of 5-FU. To avoid an influence of 5-FU, all resistant cell lines were cultured in 5-FU–free medium for over 3 weeks before subsequent analysis. For *in vitro* cytotoxicity analysis, the overnight-cultured cells (5,000/well in 96-well flat-bottomed microtiter plates) were exposed to 5-FU (Sigma, Dorset, United Kingdom) for 48 hours and released in drug-free medium for 48 hours before being subjected to a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (7).

Stable Transfection. $MCF\text{-}7_{WT}$ cells (5×10^4/well) were cultured in 35-mm dishes until 70% confluent, and Superfect (Qiagen, West Sussex,

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[3] Unpublished data.

United Kingdom) was used to cotransfect with pRcCMV/neo/nuclear factor κB (NFκB)-p50 and pcDNA3.1/Hygro/NFκB-p65, following the manufacturer's instructions. Empty vector transfected cells were used as a negative control. The successfully transfected clones were selected in G418 (500 μg/mL; for negative control cell line) or coselected in G418 (500 μg/mL) and hygromycin (150 μg/mL; for NFκB p50 + p65-cotransfected cell line). To avoid bias between individual clones, all of the selected cells were collected as a pooled population for further analysis.

Western Blot Analysis. Total protein (100 μg/lane) was electrophoresed through a 10% NuPAGE Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Millipore, Watford, United Kingdom). The blots were stained with primary antibodies (NFκB/p50 and p65, cyclin A, cyclin D3, cyclin-dependent kinase 2 (Cdk2), B-Myb, and TS, 1:250; c-FLIP, thymidine kinase (TK), and C-YES, 1:1000; α-tubulin, 1:2000) overnight at 4°C and then with horseradish peroxidase-conjugated donkey antirabbit or mouse secondary antibodies (1:5,000) for 1 hour at room temperature. The signal was detected with an ECL Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Electrophoretic Mobility-Shift Assays. Nuclear protein extraction and electrophoretic mobility-shift assays (EMSA) were carried out as described previously (8). Nuclear extract (5 μg) was incubated with 1 μg of poly(dI · dC) (Sigma) in binding buffer [50 mmol/L Tris (pH 7.6), 250 mmol/L KCl, 25 mmol/L DTT, 5 mmol/L EDTA, and 25% glycerol] for 10 minutes at room temperature. Approximately 20,000 cpm of ^{32}P-labeled 22-mer double-stranded NFκB DNA probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was added and incubated at room temperature for 20 minutes. Oct-1 (5′-TGTCGAATGCAAATCACTAGAA-3′) was used as loading control for EMSA. The EMSA conditions for Oct-1 were the same as those for NFκB. For binding specificity determination, 5 μg of nuclear extract from p50- and p65-transfected MCF-7 cells were incubated with 20 × wild-type or mutant (5′-AGTTGATATTACTTTTATAGGC-3′) unlabelled NFκB probe for 30 minutes before EMSA. The complexes were separated on a 6% polyacrylamide gel and exposed to autoradiographs.

Flow Cytometric Analysis of DNA Content. Cells were cultured in 5-FU–free medium in 25 cm flasks until 70% confluent and harvested by trypsinization. After fixation in 70% ice-cold EtOH for 10 minutes and incubation with RNase A (100 μg/mL) and propidium iodide (50 μg/mL) for 30 minutes, 10,000 cells from each sample were subjected to fluorescence-activated cell sorter (FACS) Scan (Becton Dickinson, Franklin Lakes, NJ) analysis.

Gene Expression Analysis on HG-U133A Arrays. Cells (70% confluent) cultured in 5-FU–free medium in 75 cm flasks were harvested by trypsinization. Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's protocols. Preparation of cDNA (Invitrogen) and biotin-labeled cRNA (ENZO Diagnostics, Farmingdale NY) from 8 μg of total RNA, followed by HG-U133A GeneChip hybridization, washing, staining and scanning were carried out according to the standard protocols (Affymetrix, Santa Clara, CA).

Data Transformation and Statistical Analysis. Preliminary filtering of data were done with Microarray Analysis Suite v5.0, MicroDB v3.0 and DMT v3.0 (Affymetrix). The "detection call" algorithm (Affymetrix) was used to produce a filtered gene list containing transcripts that were "present" in at least one cell line to select transcripts expressed at a level detectable on the HG-U133A arrays. The "change call" algorithm (Affymetrix) was used to further refine this gene list by selecting genes that were called "increase/marginal increase" or "decrease/marginal decrease" in all resistant cells compared with their relevant paired sensitive parent line ($n = 127$). In comparative analysis, the change call algorithm compares each probe pair within a set between experimental and baseline arrays. A change P value is calculated based on differences between perfect match (PM) and mismatch (MM) as well as between PM and background with Wilcoxon's Signed Rank test. At least four of nine cell lines expressed these 127 transcripts. Most of these transcripts were expressed in all cells ($n = 91$), with different levels of expression between sensitive and resistant pairs. This detection and change call-filtered gene list ($n = 127$) was exported into GeneSpring v6.1 for further analysis.

The signal was further normalized in GeneSpring v6.1. (*a*) Data transformation, measurements <0.01 were adjusted to 0.01 to allow more efficient analysis of log-transformed data; (*b*) per chip normalization, each measurement on an individual array was normalized to the 50th percentile of all of the measurements on the array; and (*c*) per gene to median, each gene was normalized to its median value across all arrays in the experiment, to compare the relative change in gene expression levels across different arrays.

The normalized gene expression measurements were subjected to further threshold and probability filtering in GeneSpring v6.1 to identify genes correlated with 5-FU resistance. 5-FU resistance-associated gene sets were further analyzed with unsupervised and supervised hierarchical clustering based on the standard correlation of logarithmic transformed data. Leave-one-out cross-validation based on Fishers exact t test hypergeometric probability and K nearest neighbours was used to assess the predictive power of the filtered gene sets.

Student's t test was used for other data analysis in this study.

RESULTS

5-FU Cytotoxicity in the Cancer Cell Lines. The cytotoxicity of 5-FU to the cell lines used in this study was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis (Table 1). Compared with the parental cell lines, the drug-resistant cell lines tolerate significantly higher concentrations of 5-FU (5.5- to 35.9-fold, $P < 0.01$).

Gene Expression Profiles Predictive of a 5-FU–Resistant Phenotype. Gene expression levels of all transcripts ($n = 22,283$), clustered samples according to the tissue origin of the cancer cell lines (*i.e.,* breast *versus* CRC, unsupervised hierarchical analysis, data not shown). This indicates that tissue-specific gene expression, rather than 5-FU sensitivity related expression, is the predominant transcriptome signature differentiating these cells lines.

In unsupervised analysis, threshold and probability filtering were used in DMTv3.0 and GeneSpring v6.1 software to identify genes associated with 5-FU resistance in all cancer cell lines. A set of 127 genes expressed in ≥4 cell lines and called as changed in expression between all resistant and paired parental lines was identified with the "detection and change call" algorithms in DMTv3.0 (see Materials and Methods). Forty-one of the 127 transcripts were statistically significantly associated with sensitivity to 5-FU in all cell lines (Welch's t test $P < 0.05$ with Benjamini and Hochberg false discovery rate correction). The mean expression of these 41 transcripts was altered by at least 1.5-fold between the resistant and the sensitive groups. Using a parametric model I fixed-effects 2-way ANOVA ($P < 0.05$) with Benjamini and Hochberg false discovery rate correction ($MCF\text{-}7_{FU1}$ was excluded from this analysis to balance the groups), we identified expression of 39 of these 41 genes as associated significantly with 5-FU sensitivity without substantial association with the primary tumor site (breast *versus* CRC). No interaction effect was observed between the tissue and 5-FU sensitivity phenotypic groups. This refined set of 5-FU sensitivity-associated tissue site-independent genes ($n = 39$) clustered samples according to 5-FU sensitivity class in unsupervised hierarchical analysis and successfully discriminated each individual cell line (data not shown).

The prediction strength and accuracy of the filtered gene list was estimated with leave-one-out cross-validation based on Fisher's exact test hypergeometric probability of each gene and K nearest neighbours (K = 3). A prediction rule built from the gene expression patterns of the 33 transcripts representing 29 unique genes, with the highest predictive strength (prediction strength [−ln P value] of each

Table 1 *IC_{50} (μM) of 5-FU in different cancer cell lines*

	H630	H630B	MCF-7	T47D
Parent line	30.85	16.04	1.20	2.35
Resistant line	1106.33	477.27	6.55 *	85.63
			29.33 †	

* MCF-7FU1.
† MCF-7FU5.

gene = 4.83; Table 2), discriminated 5-FU sensitivity phenotype with 100% accuracy and high predictive strength (K nearest neighbours *P* value ratio of neighborhood samples of each phenotype <0.08). These genes were able to separate cell lines according to 5-FU sensitivity phenotype with standard correlation of logarithmic-transformed gene expression data in unsupervised hierarchical clustering (Fig. 1*A*).

Supervised Analysis of Molecular Mechanisms of 5-FU Resistance. Supervised analysis of the microarray data were done to evaluate key factors hypothesized or shown previously to play a role in 5-FU metabolism or resistance. Simplified Gene Ontology (RefSeq, UniGene, and LocusLink, GeneSpring v6.1), NetAffx (Affymetrix, Santa Clara, CA), Gene Ontology biological process and Kyoto Encyclopedia of Genes and Genomes biological pathways were used to evaluate key biological pathways (NFκB, apoptotic, cell death, cell cycle, pyrimidine metabolic, DNA replication, oncogenes, tumor suppressor genes, drug resistance, and signal transduction pathways; $n = 8347$). Threshold (≥1.5-fold) and probability filtering (Welch's *t* test, $P < 0.05$) of these ontological groups (GeneSpring v6.1) identified 100 transcripts, representing 91 unique genes, the mean expression of which was consistently and significantly altered between the sensitive and resistant groups and which were called as changed in expression in at least four resistant cell lines compared with the paired parental line (change call algorithm, DMTv3.0; see Materials and Methods; Table 3 and supplementary data). Gene expression levels of these 100 transcripts separated hierarchical clustering samples into two primary groups based on 5-FU sensitivity phenotype (Fig. 1*B*).

As shown in Table 3, genes involved in 5-FU metabolism were significantly down-regulated. The products of a number of these genes (TK, orotate phosphoribosyltransferase, pyrimidine nucleoside phosphorylase, and uridine monophosphate kinase) are directly or indirectly involved in the conversion of 5-FU into its cytotoxic metabolites. TS and c-yes genes are commonly coamplified in TS inhibitor-resistant cancer cell lines. Overexpression of TS mRNA was only detected by microarray analysis in two CRC cell lines. Overexpression of a neighboring gene, C-YES was detected in all cell lines (all "Increased," change call algorithm, DMTv3.0), although it did not reach statistical significance (Table 3).

Another intriguing group is DNA replication licensing factors and cell cycle related genes. The expression of many genes governing DNA replication (*e.g.*, DNA polymerase α, MCM2, and MCM7) and those involved in G_1-S (B-Myb, cyclin D3, and Cdk2) or S-phase (cyclin A) transition was significantly down-regulated in resistant compared with sensitive lines (Table 3).

NFκB-related Genes and 5-FU Resistance. NFκB is a transcription factor that has been reported to antagonize apoptosis induced by specific cytokines and anticancer drugs (8–10). The mean expression of NFκB p65 mRNA was increased by over 1.5-fold in the resistant compared with the sensitive groups, although it did not reach statistical significance, and variable changes were observed in paired samples (Table 3). Because of the reported role of NFκB in chemoresistance, protein expression levels of NFκB p50 and p65 were analyzed in more detail. An increase in p65 protein was detected in all 5-FU–resistant cell lines compared with the sensitive parent line (Fig. 2*A*). In contrast to p65, p50 protein overexpression was only detected in one 5-FU–resistant CRC cell line (Fig. 2*A*). Consistent with our previous data, strongly enhanced NFκB DNA binding (Fig. 2*B*; ref. 11) and transcriptional activities (data not shown) were detected in all 5-FU–resistant cell lines. To determine the effect of NFκB on 5-FU resistance, the human breast cancer cell line MCF-7 was cotransfected with NFκB p50 and p65. The transfected cells showed high expression of NFκB p50 and p65 proteins (Fig. 3*A*) and stronger NFκB DNA binding (Fig. 3*B*) and transcriptional (data not shown) activities. The NFκB-transfected cells (IC_{50}, 22.5 μmol/L) are more resistant to 5-FU–induced cytotoxicity than the control cells (IC_{50}, 3.75 μmol/L; Fig. 3*C*).

C-FLIP is an antiapoptotic protein consisting of short (28 kilodaltons) and long (55 kilodaltons) isoforms (12). Consistent with c-FLIP mRNA overexpression measured in the microarray analysis (Table 3), $c\text{-}FLIP_L$ protein was overexpressed in resistant compared with the sensitive parental lines (Fig. 2*A*). Overexpression of $c\text{-}FLIP_L$ protein was more consistent and much stronger than that of $c\text{-}FLIP_S$ in resistant cell lines (Fig. 2*A*).

Down-Regulation of G_1 Checkpoint-related Genes and Delayed G_1-S Transition in 5-FU–resistant Cell Lines. Statistically significant down-regulation of cell cycle-related genes (including cyclin A, cyclin D3, Cdk2, and B-Myb) was detected in 5-FU–resistant cells by microarray analysis (Table 3). Consistent with the microarray data, cyclin A, cyclin D3, and Cdk2 proteins were down-regulated in resistant–cancer cell lines (Fig. 4*A*). Although microarray analysis showed significant down-regulation of b-Myb mRNA in all resistant cell lines, b-Myb protein was not differentially expressed between the resistant and parental cell lines.

All of the above genes are involved in G_1-S– and S-phase transition. Deregulation of some or all of them may prevent the cells from progressing into or passing through S phase, which will slow down the growth rates of the 5-FU–resistant cell lines (13). To test this hypothesis, growth rates and cell cycle profiles were determined by growth curves and FACS analysis, respectively. In comparison with the relevant parental cell lines, the 5-FU–resistant cells grew at a much slower rate (Fig. 4*B*). Compared with parental cell lines, the 5-FU–resistant cells consisted a significantly higher percentage of cells in G_1-G_0–phase and a lower percentage in S phase ($P < 0.01$; Table 4; Fig. 4*C*). These data indicate that DNA synthesis in 5-FU–resistant cells is delayed, which may be caused by deregulation of key cell cycle-related genes. BrdUrd and PI traced cell cycle analysis showed that the resistant cells were delayed in G_1 (breast cancer cell lines) or at the G_1-S boundary (H630-R10; data not shown).

NFκB-induced Arrest at G_1 Checkpoint. To test if delayed G_1-S–phase transition is a common feature of 5-FU resistance, independent of the 5-FU resistance generating mechanism, the growth rates, cell cycle profiles, and expression levels of key cell cycle-related proteins were compared between NFκB-transfected and control MCF-7 cells. The NFκB-transfected cells grew much slower than the control cells (Fig. 5*A*). FACS analysis indicated that a higher percentage of NFκB-transfected cells were in G_1-G_0 phase with a reduced population in S-phase (Fig. 5*B*). Down-regulation of key G_1-S transition-related proteins (cyclin D3, Cdk2, and B-Myb) was also detected in the transfected cells (Fig. 5*C*).

Altered Expression of Pyrimidine Metabolic Related Genes in 5-FU–resistant Cell Lines. TK converts FUdR to fluorodeoxyuridine monophosphate, a cytotoxic 5-FU metabolite. Fluorodeoxyuridine monophosphate directly inhibits TS activity and is also converted to fluorodeoxyuridine triphosphate, which is able to incorporate into and damage DNA (1). In line with our microarray data (Table 3), down-regulation of TK protein was observed in 5-FU–resistant H630 and T47D cancer cell lines (Fig. 6). However, down-regulated expression of TK mRNA in the resistant MCF-7 cells (Table 3) was not reflected at the protein level (Fig. 6).

As a key enzyme converting dUMP to dTMP, TS is essential for DNA synthesis and repair. TS protein overexpression is widely accepted as a common, although not universal, molecular feature of 5-FU resistance. Overexpression of TS mRNA was only detected in 5-FU–resistant CRC cell lines whereas all resistant cell lines expressed higher levels of TS protein (Fig. 6) indicating that the post-transcriptional mechanisms are involved in TS protein overexpression. C-yes is an oncogene located

Table 2 *5-FU sensitivity predictive gene set identified in unsupervised analysis*

Affymetrix probe set ID	Fold change *	Gene symbol	Map	Accession number	Description and functions
200734_s_at	−1.73 (−2.14 to −1.35)	ARF3	12q13	NM_001659	ADP-ribosylation factor 3; intracellular protein transport; nonselective vesicle assembly; small GTPase-mediated signal transduction
201008_s_at † 201009_s_at	4.73 (2.6–6.55)	TXNIP	1q21.2	NM_006472	Thioredoxin interacting protein; signal transduction
201093_x_at † 222021_x_at	−3.07 (−5.92 to −2)	SDHA	5p15	NM_004168	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp); Tricarboxylic acid cycle
201678_s_at	−2.81 (−4.26 to −1.32)	DC12	3q21.3	NM_020187	DC12 protein
201695_s_at	−3.53 (−5.24 to −2.32)	NP	14q13.1	NM_000270	Nucleoside phosphorylase; purine metabolism; pyrimidine metabolism; nicotinate and nicotinamide metabolism
201710_at	−3.9 (−4.42 to −3.15)	MYBL2	20q13.1	NM_002466	v-myb myeloblastosis viral oncogene homolog (avian)-like 2; regulation of cell cycle; regulation of transcription, DNA-dependent; transcription from Pol II promoter; antiapoptosis; development
202338_at	−2.74 (−5.2 to −1.5)	TK1	17q23.2-q25.3	NM_003258	Thymidine kinase 1, soluble; DNA metabolism
202392_s_at	2.50 (1.58–4.07)	PISD	22q12.2	NM_014338	Phosphatidylserine decarboxylase; phospholipid biosynthesis
202406_s_at	−2.02 (−3.46 to −1.3)	TIAL1	10q	NM_003252	TIA1 cytotoxic granule-associated RNA binding protein-like 1; regulation of transcription from Pol II promoter; induction of apoptosis
202735_at† 213787_s_at	−2.30 (−3.77 to −1.34)	EBP	Xp11.23-p11.22	NM_006579	Emopamil binding protein (sterol isomerase); skeletal development; cholesterol biosynthesis
202942_at	−3.57 (−7.05 to −2.4)	ETFB	19q13.3	NM_001985	Electron-transfer-flavoprotein, β polypeptide; electron transport
203046_s_at	−2.56 (−3.19 to −1.74)	TIMELESS	12q12-q13	NM_003920	Timeless homolog (Drosophila)
203564_at	−2.62 (−3.62 to −1.47)	FANCG	9p13	NM_004629	Fanconi anemia, complementation group G; cell cycle checkpoint; DNA repair
203633_at	−1.53 (−1.83 to −1.34)	CPT1A	11q13.1-q13.2	NM_001876	Carnitine palmitoyltransferase 1A (liver); fatty acid metabolism; fatty acid β-oxidation; transport
203696_s_at	−2.15 (−3.89 to −1.18)	RFC2	7q11.23	NM_002914	Replication factor C (activator 1) 2, 40 kDa; DNA replication
207980_s_at 209357_at †	4.98 (2.66–7.94)	CITED2	6q23.3	NM_006079	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2; regulation of transcription from Pol II promoter
208002_s_at	−2.57 (−4.11 to −1.76)	BACH	1p36.31-p36.11	NM_007274	Brain acyl-CoA hydrolase; lipid metabolism
208785_s_at	1.78 (1.29–2.18)	—	—	AF183417	Strong similarity to protein microtubule-associated proteins 1A/1B light chain 3 (*Homo sapiens*)
209014_at	−1.72 (−2.18 to −1.37)	MAGED1	Xp11.23	NM_006986	Melanoma antigen, family D, 1
209605_at	−3.85 (−5.03 to −1.48)	TST	22q13.1	NM_003312	Thiosulfate sulfurtransferase (rhodanese); sulfate transport; cyanate catabolism
213008_at	−3.65 (−8.1 to −1.74)	FLJ10719	15q25-q26	BG403615	Hypothetical protein FLJ10719
213546_at	1.91 (1.43–3.06)	DKFZp586I1420	7p15.1	NM_152747	Hypothetical protein DKFZp586I1420
213726_x_at	−1.97 (−2.45 to −1.37)	TUBB2	—	NM_006088	Tubulin, β, 2; microtubule-based movement; natural killer cell mediated cytolysis
214143_x_at	−1.65 (−2.11 to −1.38)	RPL24	3q12	NM_000986	Ribosomal protein L24; protein biosynthesis
216250_s_at	2.33 (1.91–2.77)	LPXN	11q12.1	NM_004811	Leupaxin; protein complex assembly; cell adhesion; signal transduction
217860_at	−1.61 (−1.84 to −1.23)	NDUFA10	2q37.3	NM_004544	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 10, 42 kDa; energy pathways
218883_s_at	−2.80 (−4.63 to −1.5)	KLIP1	4q35.1	NM_024629	KSHV latent nuclear antigen interacting protein 1
220761_s_at	2.85 (1.47–3.77)	JIK	12q	NM_016281	STE20-like kinase; protein amino acid phosphorylation; JNK cascade
38158_at	−4.01 (−7.72 to −1.93)	ESPL1	12q13.3	NM_012291	Extra spindle poles like 1 (*S. cerevisiae*); regulation of cell cycle

Abbreviation: ID, identification; KSHV, Kaposi's sarcoma-associated herpesvirus; JNK, c-Jun NH_2-terminal kinase; —, data not available.
* Mean fold change of resistant/sensitive signal. Negative values, down-regulation; figures in parentheses, range of fold change in paired cell lines.
† The probe set used to indicate the fold change.

adjacent to TS. We have reported previously that c-yes and TS genes are coamplified and overexpressed in TS inhibitor-resistant cancer cell lines (4). In this study, overexpression of C-YES protein (Fig. 6) and mRNA (Table 3) was consistently detected in 5-FU–resistant cell lines. However, the weakest up-regulation of c-yes mRNA, which was observed in the $MCF7_{FU1}$-resistant cells, was not detected at the protein level. Another adjacent gene, which regulates TS expression (rTS), was overexpressed in three resistant cell lines compared with the paired sensitive parent (Table 3).

DISCUSSION

Affymetrix HG-U133A oligonucleotide microarrays were used to analyze a panel of five 5-FU–resistant and paired sensitive parental

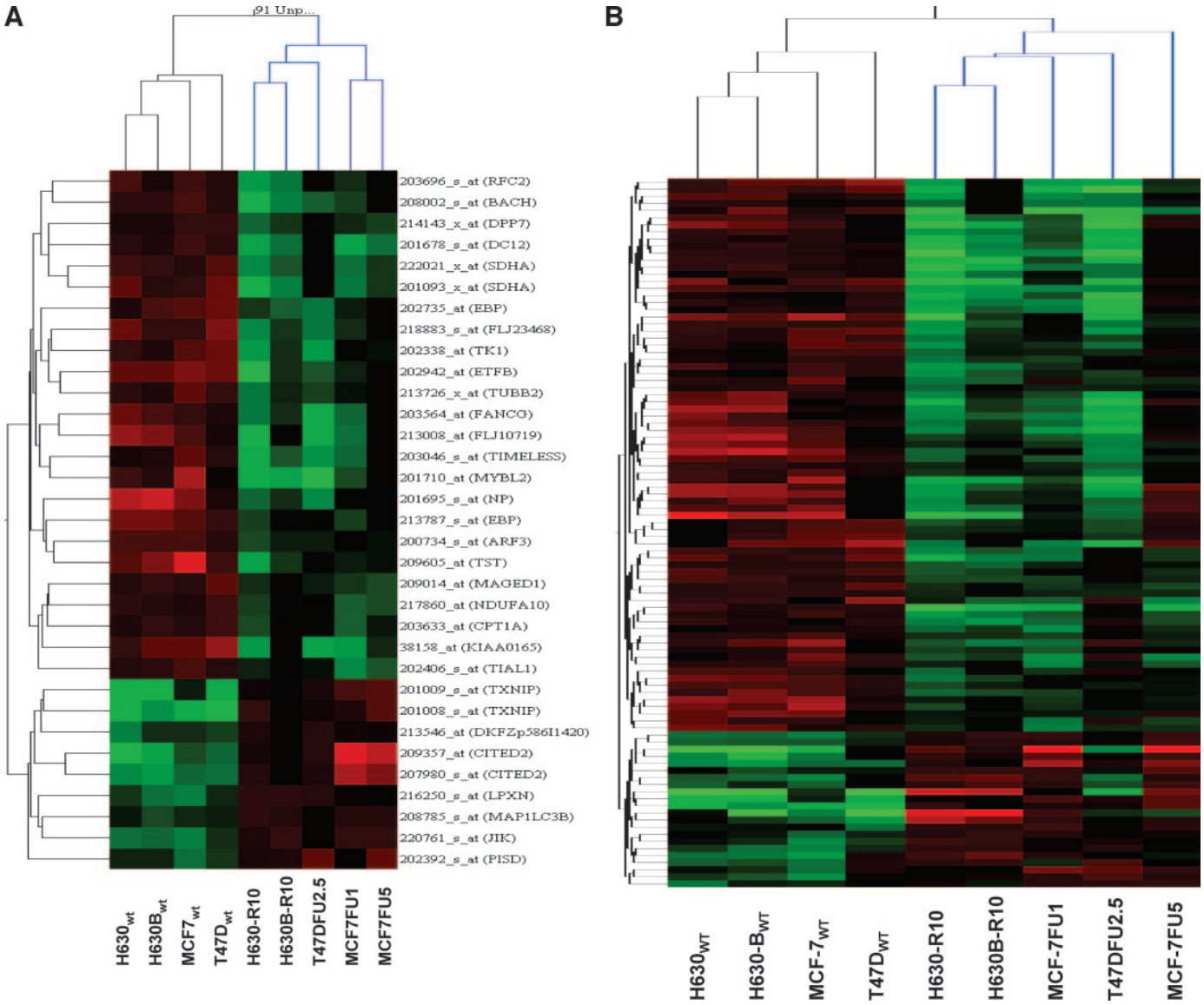

Fig. 1. Two-dimensional hierarchical clustering analysis of gene expression profiles in nine cancer cell lines. *Color matrix* and *dendrograms* illustrating standard correlation of log-transformed gene expression profiles of the following: (*A*) unsupervised analysis, 33 transcripts were associated with and predictive of 5-FU sensitivity independent of the tissue of origin of the cancer cell lines, and (*B*) supervised analysis, 100 transcripts significantly and consistently associated with 5-FU sensitivity phenotype. *Horizontal axis* represents cell lines; two major clusters, sensitive parental cell lines (*gray*), and resistant daughter cell lines (*blue*). *Vertical axis* represents genes. *Color bars*, *red*, up-regulated; *green*, down-regulated; *black*, no change. Affymetrix probe identifications and gene names are shown to the right of *A*.

cell lines. In unsupervised analysis, 33 5-FU sensitivity phenotype-associated transcripts representing 29 unique human genes were identified. Using the expression pattern of these genes, we predicted 5-FU sensitivity with 100% accuracy and high predictive strength. The strength and accuracy of this gene set for prediction of 5-FU sensitivity will be evaluated in future studies with independent test samples, including cell line models and colorectal tumors from patients who have received 5-FU therapy.

Maxwell *et al.* (14) detected 5-FU–inducible genes (SSAT, annexin II, thymosin-β-10, MAT-8 and chaperonin-10) in 5-FU–treated MCF-7 cells, and enhanced basal expression of these genes in resistant H630-R10 cells. Enhanced basal expression of 5-FU–inducible genes in resistant cells is not associated with increased cell cycle arrest or apoptosis (14). Overexpression of most of these genes (Annexin II, MAT-8, and thymosin 10) was also detected in H630-R10 cells in our study (data not shown). The predictive markers may not necessarily represent molecules critical to the acquisition or maintenance of the resistant phenotype (4, 14). Some or all of these genes may be markers of the phenotype that have no role in resistance (*e.g.*, coamplified genes) or they may represent the amplified end points of critical cellular pathways, in which the upstream mediators of the resistant phenotype undergo small but critical expression changes.

Supervised analysis of the microarray data using gene ontologies was used to examine pathways and factors shown previously or hypothesized to play a role in 5-FU metabolism or resistance. This analysis identified 100 transcripts representing 91 unique human genes the mean expression of which was statistically significantly altered in 5-FU–resistant cell lines compared with sensitive parental cell lines by $\geq$1.5-fold, and the expression of which was either increased or decreased in at least four of the resistant cell lines compared with the paired parental line.

We verified the protein expression levels of five of these genes using Western blot. The protein and mRNA expression patterns from all except one (b-myb) of the tested genes were either correlated in all cell lines (cyclin A, cyclin D3, and cdk2) or correlated in both the CRC but only one breast cancer cell line (TK). For the gene where

Table 3 *5-FU resistance associated genes identified in supervised analysis*

Affymetrix probe set ID	Fold change	Gene symbol	Accession number	Map	Description and functions
NFκB pathway and apoptosis-related genes					
204798_at	−6.21 * (−1.76 to −35.81)	MYB	NM_005375	6q22-q23	c-Myb oncogene which influences angiogenesis, proliferation and apoptosis.
204825_at	−2.92 (−5.73–1.00)	MELK	NM_014791	9p11.2	A member of AMP-activated protein kinase family which can inhibits cancer growth and induce apoptosis.
202406_s_at	−1.98 (−1.45 to −3.91)	TIAL1	NM_003252	10q	Regulation of transcription from Pol II promoter, induction of apoptosis.
209878_s_at	1.19 (−1.50–2.07)	p65/RelA †	M62399	11q13	A major subunit of NFκB family.
208485_x_at	2.35 (1.07–3.57)	FLIP †	NM_003879	2q33-q34	FLICE-like inhibitory protein; forming stable complexes with Fas, FADD, and caspase 8 to prevent the autoproteolytic cleavage and activation of caspase 8.
Cell cycle-related genes					
203418_at	−4.62 (−1.22 to −19.18)	CCN1	NM_001237	4q25-q31	Cyclin A, an important regulator for maintaining S-phase progression.
201710_at	−3.74 (−3.28 to −5.19)	BMYB	NM_002466	20q13.1	B-Myb, a regulator for transition from G_1 to S phase.
201457_x_at	−2.49 (−1.35 to −4.76)	BUB3	NM_004725	10q26	Mitosis, cell proliferation.
205167_s_at	−3.07 (−1.13 to −13.05)	CDC25C	NM_001790 NM_022809	5q31	Regulation of CDK activity and mitosis, dephosphorylation.
204252_at	−2.58 (−2.11 to −3.54)	p33(CDK2)	M68520	12q13	Cdk2, phosphorylation of pRb by Cdk2 is the penultimate step in the transition from G_1 to S phase.
201700_at	−1.94 (−1.36 to −2.57)	CCND3	NM_001760	6p21	Cyclin D3, a regulator for transition from G_1 to S phase.
201395_at	1.79 (−1.52–2.72)	RBM5	NM_005778	3p21.3	RNA processing, negative regulation of cell cycle.
Pyrimidine metabolic and DNA replication-related genes					
209773_s_at	−4.99 (−1.21 to −18.66)	RRM2	NM_001034	2p25-p24	DNA replication and deoxyribonucleoside diphosphate metabolism.
201695_s_at	−3.65 (−2.63 to −6.22)	PNP	NM_000270	14q13.1	Purine nucleoside phosphorylase, high level of PNP sensitises cancer cells to 5-FU.
202107_s_at	−3.62 (−1.72 to −5.88)	MCM2	NM_004526	3q21	Minichromosome maintenance 2 protein, DNA replication licensing factor, and a marker of proliferating cells.
208795_s_at	−3.14 (−1.53 to −8.14)	MCM7	NM_005916 NM_182776	7q21.3-q22.1	Minichromosome maintenance 7 protein, DNA replication licensing factor, and a marker of proliferating cells.
204127_at	−3.22 (−1.08 to −7.89)	RFC3	NM_002915 NM_181558	13q12.3- q13	DNA replication and DNA chain elongation.
205053_at	−3.34 (−1.42 to −4.84)	PRIM1	NM_000946	12q13	DNA replication priming.
212020_s_at ‡	−2.61	MKI67	NM_002417	10q25-q^{ter}	Ki-67 antigen, expressed in proliferating cells
212021_s_at	−1.33 to −5.60				
209825_s_at	−2.93 (−1.13 to −7.12)	UMPK	BC002906	1p32	Uridine monophosphate kinase, converting FUR to FUMP, an essential and rate-limiting enzyme in 5-FU activation.
209408_at	−3.30	KIF2C	NM_006845	1p34.1	Mitosis and cell proliferation.
211519_s_at ‡	(−1.33 to −6.30)				
202338_at	−2.64 (−1.61 to −5.71)	TK1	NM_003258	17q23.2-q25.3	Thymidine kinase 1, converting FdUrd to FdUMP.
202726_at	−2.26 (−1.38 to −4.92)	LIG1	NM_000234	19q13.2- q13.3	DNA replication, repair, and recombination.
204441_s_at	−2.25 (−1.22 to −3.51)	POLA2	NM_002689	11q13.1	DNA poly merase α, The only enzyme in eukaryotic cells capable of starting DNA chains *de novo*.
215165_s_at	−2.41 (−1.38 to −4.72)	OPRT	D86227	3q13	Orotate phosphoribosyl transferase, converting 5-FU to FUMP.
213007_at	−2.42 (−1.43 to −3.91)	POLG	NM_002693	15q25	Purine metabolism, pyrimidine metabolism, DNA polymerase.
203536_s_at	−1.97 (−1.17 to −2.75)	CIAO1	NM_004804	2q11.2	Regulation of transcription from Pol II promoter, positive regulation of cell proliferation.
202633_at	−2.28 (−1.49 to −3.00)	TOPBP1	NM_007027	3q22.1	DNA replication and metabolism.
204143_at	3.25 (1.33–5.47)	HSRTSBETA †	NM_017512	18p11.32	rTS gene, regulating TS expression
202932_at	2.36 (1.08–4.24)	YES1 †	NM_005433	18p11.32	C-YES gene
207980_s_at	3.52 (2.27–5.94)	CITED2	NM_006079	6q23.3	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2.
201577_at	−1.76 (−1.39 to −2.30)	NME1	NM_000269 NM_198175	17q21.3	GTP, UTP, and CTP biosynthesis
204817_at 38158_at	−3.96 (−2.08 to −8.47)	ESPL1	NM_012291	8	Regulation of cell cycle, proteolysis, apoptosis.

Abbreviations: ID, identification; OPRT, orotate phosphoribosyltransferase; FUMP, 5-fluorouridine monophosphate; FdURd, 5-fluoro-2′-deoxyuridine; FUR, fluorouridine; FADD, Fas-associated death domain; PNP, pyrimidine nucleoside phosphorylase; FdUMP, fluorodeoxyuridine monophosphate; UMPK, uridine monophosphate kinase.

* Mean fold change of resistant/sensitive signal. Negative values, down-regulation; figures in parentheses, range of fold change in paired cell lines.

† Altered but not statistically significant.

‡ The probe set used to indicate the fold change.

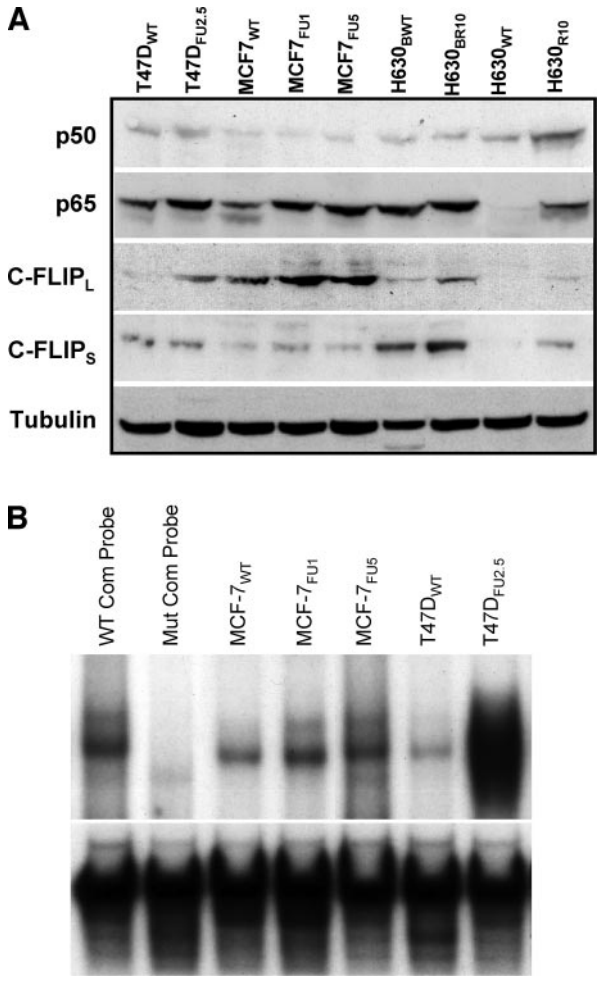

Fig. 2. Protein expression levels and DNA-binding activity of NFκB and related genes in 5-FU–resistant and –sensitive cell lines. *A*, Western blotting analysis of NFκB p50, p65, and c-FLIP expression levels. Tubulin, loading control; c-FLIP$_L$ and c-FLIP$_S$ represent long and short isoforms of c-FLIP, respectively. *B*, EMSA analysis of NFκB DNA-binding activity in 5-FU–resistant cell lines. Oct-1, loading control; WT Com and Mut Com represent wild-type and mutant competition probes, respectively.

expression of mRNA and protein was not correlated (b-Myb), post-transcriptional and/or post-translational mechanisms of regulation are likely to exist. The protein expression levels of an additional five genes, the expression of which was altered in some or all sensitive/resistant pairs (change call algorithm, DMTv3.0), but the mean expression of which was not statistically significantly different between the resistant and sensitive groups, were also evaluated. The expression of three of these genes was correlated at the mRNA and protein level in 40% (p65 and TS) to 80% (c-yes and c-Flip$_L$) of the cell lines, indicating post-transcriptional mechanisms of regulation exist in some cell lines. The change in expression of c-Flip$_S$ mRNA was correlated with protein expression changes in all cell lines. Thus, there was a strong correlation between microarray and protein data for at least 70% of the genes evaluated. The importance of a strong correlation existing between the mRNA and protein expression levels depends on both the cellular function of the factor under investigation as well as the question being addressed. To gain insight into the molecular mechanisms of the phenotype, the status of the protein product is critical.

NFκB p65 mRNA and protein overexpression were detected in the 5-FU–resistant cancer cell lines. The resistant cell lines had higher NFκB DNA binding and transcriptional activity. Inhibition of NFκB activity by IκBα can enhance the cytotoxicity of some anticancer drugs *in vitro* (8) and *in vivo* (9, 15). We have reported previously that TS inhibitor (5-FU and TDX)-resistant cancer cell lines possess high NFκB nuclear activity (11). Disulfiram, an antialcoholism drug, inhibits NFκB activity and sensitizes cancer cells to 5-FU–induced apoptosis (16). The most common NFκB dimer is a p50-p65 heterodimer. To elucidate the role of NFκB in 5-FU resistance, MCF-7 cells were cotransfected with NFκB p50 and p65. The IC_{50} of 5-FU in NFκB-transfected cells was 5.5-fold higher than the control cells. These data indicate that NFκB can induce 5-FU resistance.

In this study, altered expression of key antiapoptotic genes (*e.g.*, *c-IAPs*, *XIAP*, *A1/Bfl-1*, and *IEX-1L*) downstream of NFκB (17–19) was evaluated. Expression of c-IAP-1 was increased in all except one (T47D) resistant line (1.5–2.1 fold). This change did not reach statistical significance in the supervised analysis. There was a large increase in c-IAP-2 mRNA expression in resistant CRC cells lines (H630-R10, 11.0-fold and H630-R10-B 6.6-fold) but not in breast cells. Because of the nature of the probe design, the two isoforms of IEX-1 (antiapoptotic IEX-1L and proapoptotic IEX-1S) are represented by a single probe set on the HG-U133A array, making interpretation of the data difficult. IEX-1 was decreased in all three resistant breast cancer cell lines (−1.6- to −2.3-fold), and its expression was either slightly increased (H630-R10 1.4-fold) or unaltered (H630B-R10) in the resistant CRC cell lines. Bfl-1/A1 and XIAP mRNA were not expressed in any of the cells under investigation.

c-FLIP is another antiapoptotic gene, the mRNA and protein of which were overexpressed in some 5-FU–resistant cell lines (Fig. 2*A*;

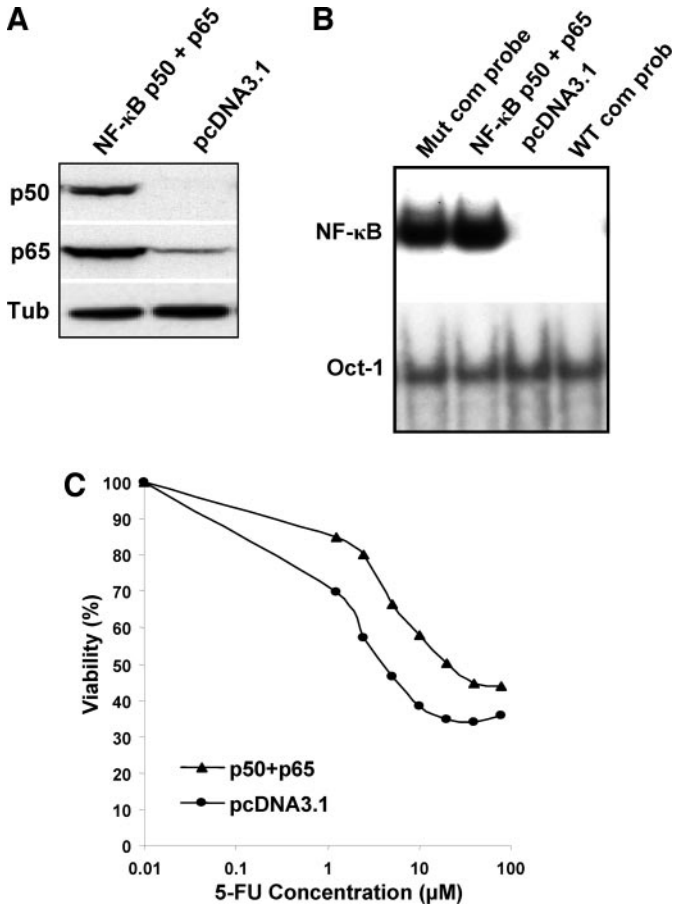

Fig. 3. NFκB-transfected MCF-7 cells. *A*, Western analysis of NF-κB p50 and p65 expression levels in NFκB-transfected and control MCF-7 cells. *B*, NFκB DNA-binding activity in NFκB-transfected and control MCF-7 cells. *C*, cytotoxicity of 5-FU to NFκB-transfected and control MCF-7 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method was used to analyze cell viability.

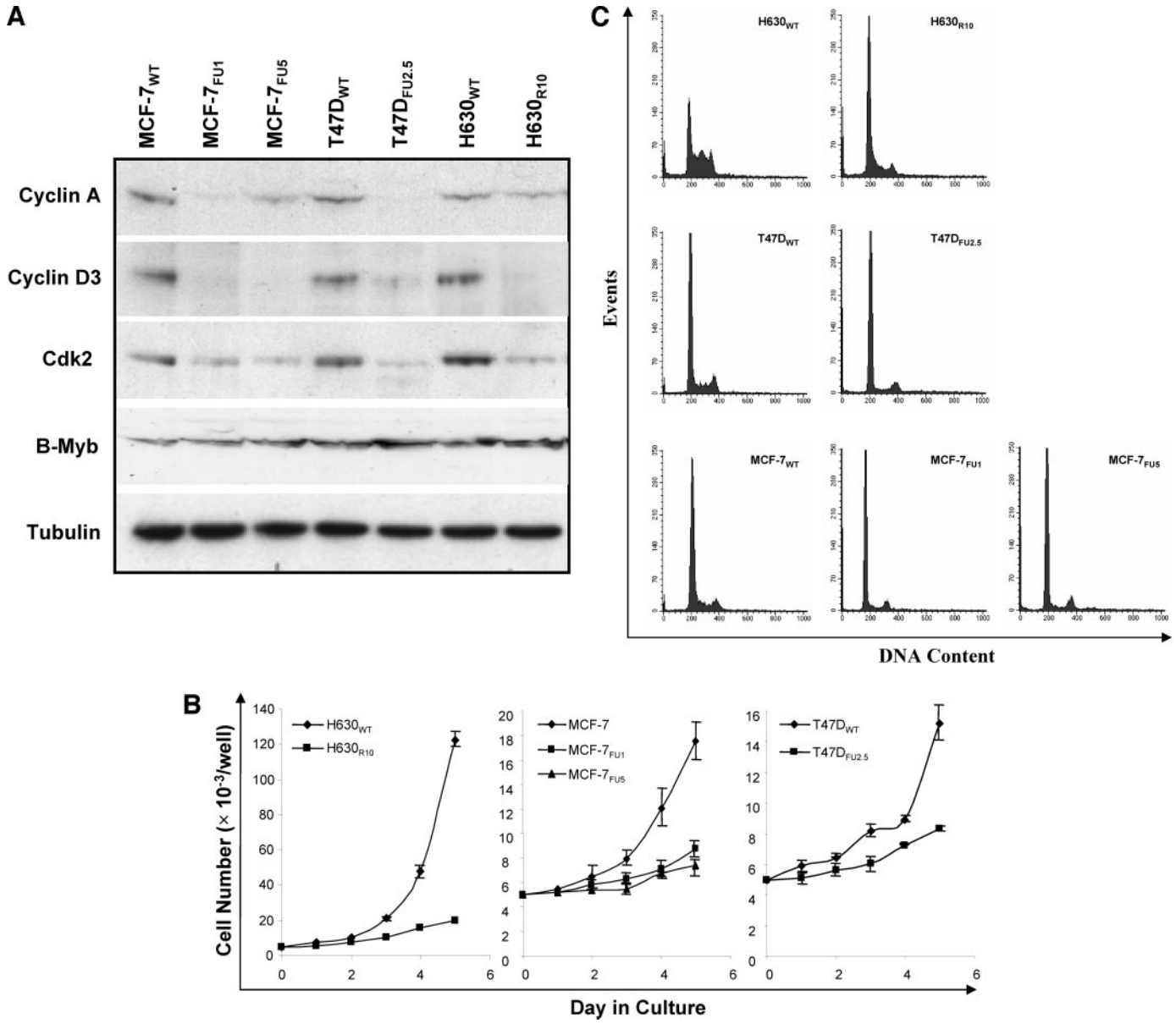

Fig. 4. Cell proliferation status and G_1-S transition-related protein expression levels in 5-FU–resistant and –sensitive cell lines. *A*, Western blot of the expression levels of key G_1-S transition-related proteins. Tubulin, loading control. *B*, compared with parental cell lines, 5-FU–resistant cell lines grew significantly slower. Mean and SD of triplicate experiments are shown. *C*, representative FACS histograms of DNA content in different cell lines. Triplicate experiments were done (see Table 4).

Table 3). c-FLIP encodes short (c-$FLIP_S$) and long (c-$FLIP_L$) isoforms (12). The c-$FLIP_L$ protein was more significantly and consistently up-regulated in all resistant cancer cell lines (Fig. 2*A*). c-FLIP inhibits cleavage and activation of caspase 8, the initiator caspase of the extrinsic apoptotic pathway (20). It has been reported that 5-FU transcriptionally induces CD95 expression, which triggers caspase 8 activation and apoptosis via the extrinsic apoptotic pathway (21, 22). 5-FU also inhibits c-FLIP expression in cancer cell lines (23). High c-FLIP expression in 5-FU–resistant cell lines may block the extrinsic apoptotic pathway and inhibit the cytotoxicity of 5-FU in the resistant cells. c-FLIP can be up-regulated by NFκB and c-FLIP protein overexpression can replace NFκB to block the tumor necrosis factor α-induced extrinsic apoptotic pathway activation in human cancer cell lines (24, 25). C-Flip gene expression is 5-FU inducible (14). We are currently investigating the relationship between NFκB and c-FLIP in 5-FU resistance.

In cancer cells, 5-FU is converted to several active metabolites intracellularly (fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate, and fluorouridine triphosphate) that are cytotoxic (1). There have been several reports demonstrating the relationship of

Table 4 *Percentage of cells in different cell cycle phases*

	MCF-7	MCF-7_{FU1}	MCF-7_{FU5}	T47D	T47D$_{FU2.5}$	H630$_{WT}$	H630$_{R10}$
G_0/G_1	74.81 (0.15)	90.44 (1.37)	87.68 (0.15)	75.17 (0.95)	87.70 (0.87)	56.61 (0.73)	64.20 (0.28)
S	11.19 (0.42)	2.15 (0.31)	2.93 (0.11)	10.87 (0.51)	3.43 (0.39)	30.84 (1.13)	13.28 (0.59)
G_2/M	14.00 (0.35)	7.41 (1.18)	9.40 (0.12)	13.96 (0.53)	8.87 (1.00)	12.55 (0.64)	22.52 (0.61)

Percentage of cells in each phase of the cell cycle calculated from DNA histograms generated in FACS analysis of the sensitive and resistant cell lines. Mean from three replicates is shown with the SD in parentheses.

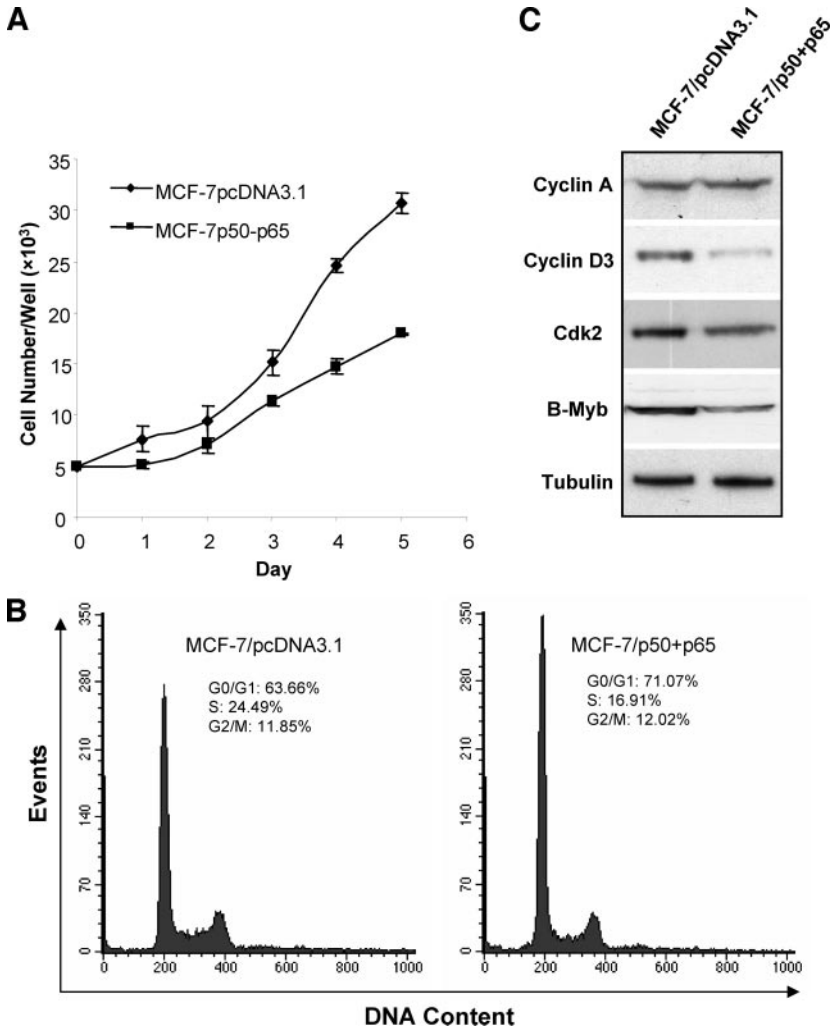

Fig. 5. Cell proliferation status and G_1-S transition-related protein expression levels in NF-κB p50/p65-transfected and control cell lines. *A*, NF-κB p50/p65-transfected MCF-7 cells grew slower than the control cells. Mean and SD of triplicate experiments are shown. *B*, representative FACS histograms of DNA content in NFκB-transfected and control cells. Triplicate experiments were done. *C*, Western analysis of expression levels of some G_1-S transition-related proteins. Tubulin, loading control.

disturbance of individual enzymes involved in 5-FU metabolism with 5-FU resistance (26, 27). In supervised analysis of the microarray data, down-regulation of several key enzymes involved in 5-FU activation (TK, orotate phosphoribosyltransferase, uridine monophosphate kinase, and pyrimidine nucleoside phosphorylase) was identified in 5-FU–resistant cell lines. Down-regulation of these pyrimidine metabolic enzymes may represent one of the pivotal self-protective mechanisms sheltering resistant cells from the cytotoxic effects of 5-FU.

5-FU is an indirect TS inhibitor. TS gene amplification and overexpression are widely accepted as a common, although not universal, feature of 5-FU resistance (1). In this study, TS protein overexpression was detected in all resistant cell lines but only resistant CRC cell lines expressed higher levels of TS mRNA. In line with previous findings (4, 28), overexpression of genes closely located to TS (rTS mRNA and C-YES mRNA and protein) was detected in resistant cell lines.

Unrestricted proliferation induced by breached G_1 checkpoint is a fundamental characteristic of cancer cells (13, 29, 30). This feature is also considered an "Achilles heel" of cancer cells, allowing some S-phase-specific anticancer drugs (*e.g.*, 5-FU and TDX) to selectively target cancer cells. In this study, 5-FU–resistant cells grew at much slower rates than the relevant parental cell lines *in vitro*. A higher percentage of resistant cells were retained in G_0-G_1 phase or at the G_1-S boundary. In line with this phenotype, down-regulation of G_1-S (cyclin D3 and Cdk2) and S-phase (cyclin A) transition-related genes was detected in 5-FU–resistant cells. The 5-FU–resistant cells also showed reduced Cdk2 kinase activity and hypophosphorylated Rb protein (data not shown). The cyclin D-Cdk4/6-cyclin E-Cdk2-Rb-E2F axis plays a pivotal role in G_1-S transition (31–34). Our findings indicated that 5-FU–resistant cells have an increased G_1 checkpoint stringency, which significantly delays the transition of resistant cells from G_1 into S-phase. Reduced expression of some DNA replication

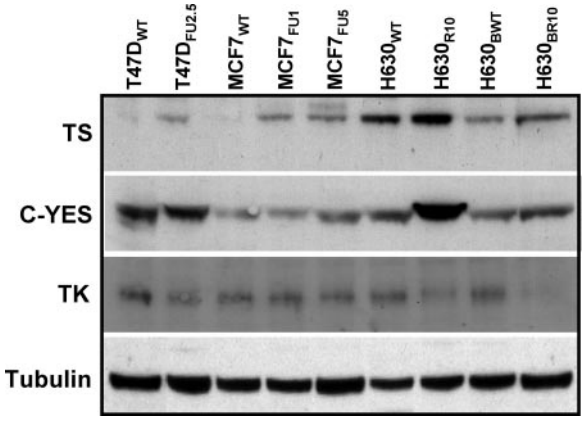

Fig. 6. Western blotting analysis of TS, C-YES, and TK expression levels. TS and C-YES proteins were consistently overexpressed, and TK was down-regulated in some 5-FU–resistant cancer cell lines. Tubulin, loading control.

licensing factors (MCM2 and 7) and DNA replication or repair-related genes (Ki-67, DNA polymerase α, TK, and CITED2) was also detected in 5-FU–resistant cell lines. 5-FU is an S-phase-specific anticancer drug. It has been reported that reduced proliferation rate is inversely correlated with 5-FU cytotoxicity and therapeutic response (35–37). A reduced proliferation rate may protect 5-FU–resistant cancer cells from the lethal attack of 5-FU. In addition, delayed S-phase entry and/or reduced S-phase traverse may provide resistant cells with enough time to repair 5-FU–induced damage before progressing to G_2-M phase.

NFκB transfection induced 5-FU resistance in MCF-7 cells. Unexpectedly, the NFκB induced 5-FU–resistant cells also showed slower growth rates and increased G_1 checkpoint stringency with reduced expression of G_1-S and S-phase transition-related genes. This phenomenon indicates that increased G_1 checkpoint stringency may be a common mechanism involved in 5-FU resistance. If this is the case, 5-FU resistance may be, at least partially, reversed by specific targeting of the G_1-S checkpoint arrest in the resistant cells. Restoration of the G_1 checkpoint by targeting Cdk2 and/or Cdk4 is currently one of the major strategies for anticancer drug development (30). Our findings indicate that within the complex genetic background of a cancer cell, G_1 checkpoint restoration may produce slowly growing cancer cells which are resistant to S-phase-specific anticancer drugs.

In conclusion, global transcriptome profiling shows that 5-FU resistance is multifactorial and involves some or all of the following cellular pathways: overproduction of 5-FU targets; up-regulation of specific antiapoptotic proteins, reduced production of 5-FU–activating enzymes, and increased G_1 checkpoint stringency with a reduced cell proliferation rate and reduction in DNA-synthetic machinery.

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