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Molecular Pharmacology of Cancer Therapy in Human Colorectal Cancer by Gene Expression Profiling[1,2]

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ABSTRACT

Global gene expression profiling has potential for elucidating the complex cellular effects and mechanisms of action of novel targeted anticancer agents or existing chemotherapeutics for which the precise molecular mechanism of action may be unclear. In this study, decreased expression of genes required for RNA and protein synthesis, and for metabolism were detected in rectal cancer biopsies taken from patients during a 5-fluorouracil infusion. Our observations demonstrate that this approach is feasible and can detect responses that may have otherwise been missed by conventional methods. The results suggested new mechanism-based combination treatments for colorectal cancer and demonstrated that expression profiling could provide valuable information on the molecular pharmacology of established and novel drugs.

INTRODUCTION

The sequencing and annotation of the human genome together with recent technological advances have enabled rapid parallel analysis of the expression of thousands of genes using arrays of oligonucleotides or cDNAs (reviewed in Refs. 1, 2). Subsequent key studies have demonstrated that global gene expression profiling can be used to classify cancers and in some instances to predict outcome (reviewed in Refs. 1, 2). Expression profiling also has great potential for understanding the mechanisms of action of both novel targeted anticancer agents and of existing therapeutics for which the precise molecular mechanism of action may not be clearly defined (1). Moreover, for both conventional and novel anticancer agents it is clear that the early identification of potential "on-target" *versus* "off-target" effects, the discovery of robust pharmacodynamic markers and the identification of patients most likely to benefit from particular regimens is becoming increasingly important. Currently, the application of global gene expression profiling to these areas has been quite limited. Initial studies have demonstrated correlations between basal gene expression and drug sensitivity in cell lines and xenograft models (3, 4), whereas others have profiled transient changes in expression after *in vitro* drug treatment (1). However, to our knowledge none of the genome-wide expression profiling studies reported currently in the literature have specifically examined the effects of treatment on tumor gene expression profile in patients during the actual period of drug exposure.

We identified a collection of serial samples, taken pre- and during treatment, from a consecutive series of patients with locally advanced rectal cancer. Using this collection we set out to explore the potential for the application of gene expression profiling by microarray to the measurement of molecular pharmacodynamic responses after treatment with a single dose of MMC[4] and during a continuous infusion of 5FU. In this study, we report for the first time gene expression profiling in cancer patients before, and critically, during the period of exposure to chemotherapy. We have demonstrated that the approach is feasible, and we have detected a novel molecular response that would not have been predicted from *in vitro* studies and that would have otherwise been missed by conventional approaches. The results also suggest a possible new therapeutic approach. Overall our observations suggest that gene expression profiling in response to treatment could greatly benefit clinical studies of conventional or novel chemotherapeutic agents and provide encouragement for additional applications of the technology in the molecular pharmacology of cancer therapy.

MATERIALS AND METHODS

Patient Details. Consecutive patients with rectal cancer referred to the Royal Marsden Hospital NHS Trust were recruited for preoperative chemoradiotherapy if there was concern about adequate surgical clearance (Table 1). Scientific and Ethical Committee approval was obtained. Pelvic MRI was used to determine T stage, and systemic spread was assessed by CT (5). All of the patients were T3 or T4 and had a significant risk of incomplete surgical clearance. Protracted venous infusional 5FU (300 mg/m^2/day) was delivered via a Hickman line for 12 weeks. MMC (7 mg/m^2) was administered as a single dose at the start of treatment and at 6 weeks. Chemoradiotherapy was undertaken for an additional 6 weeks with 5FU (200 mg/m^2/day) and radiotherapy using a CT-planned, conformally blocked, three-field technique. Phase I delivered 25 fractions of 1.8 Gy to a total of 45 Gy, with Phase II aiming to deliver a boost to the tumor of 9 Gy in 5 fractions. After chemoradiotherapy 11 patients underwent surgery 4–10 weeks after completion of radiotherapy (median 6 weeks).

Radiological assessment of response, by two independent radiologists, was determined by sequential MRI according to WHO criteria (6). The radiologists were unaware of the microarray data but were aware that the patients were undergoing chemoradiotherapy. The product of the length of the tumor and the maximal tumor thickness (at an anatomically consistent point in subsequent scans) was calculated for each MRI. Complete response was defined as disappearance of the tumor, and partial response was defined as a decrease of 50% in the product size, with progressive disease being an increase in size of $>$25% or the appearance of new lesions. Stable disease met neither partial response nor progressive disease criteria. Response of primary tumor is included for reference to the expression profiling data and was determined from MRI scans pretreatment and after 12 weeks chemotherapy. Overall responses were determined from comparison of pretreatment and postchemoradiotherapy MRI scans, and patients were classified either as responders (complete and partial responses) or nonresponders (stable and progressive disease). Final postoperative T stage was determined by histopathology of the resected tumor.

Tumors were biopsied by rigid sigmoidoscope, under direct vision, and were immediately snap-frozen and stored in liquid nitrogen. Biopsies were performed 6 weeks into the continuous infusion of 5FU and before the second dose of MMC and subsequent chemoradiotherapy. Paired pre- and during-treatment samples were obtained from 15 patients. One patient was limited to a pretreatment sample (patient 14), and 2 additional patients, treated with irinotecan (360 mg/m^2; patient 13) or raltitrexed (5.1 mg/m^2; patient 17) as a single dose every 3 weeks, were also included.

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[4] The abbreviations used are: MMC, mitomycin c; 5FU, 5-fluorouracil; SAM, significance analysis of microarrays; TS, thymidylate synthetase; MRI, magnetic resonance imaging; CT, computed tomography; HUVEC, human umbilical vascular endothelial cell; FdUMP, 5-fluoro-dUMP; HSP, heat shock protein; FdUTP, 5-fluoro-dUTP; FUTP, 5-fluoro-UTP.

Table 1 *Patient charateristics and response to treatment*

Tumors were staged at presentation using the TMN (6) system by pelvic MRI and after surgery (final stage) by histopathology. Response of the tumor to the primary chemotherapy (Resp. to chemo. column), prior to chemoradiotherapy, is included for reference to the expression profiling data (CR = complete response, PR = partial response, SD = stable disease and PD = progressive disease). Resp. to chemoRT = overall response after completion of chemoradiotherapy. (* = inoperable tumor, # = no operation, R = response and NR = non-response.) LN = lymph node involvement (− = no lymph node involvement, + = lymph node involvement). p53 and Sensc. = immunohistochemical staining for p53 or senescence-associated β-galactosidase (N = negative, P = positive and nd = not done), data from immunoblotting is also included, in brackets, for p53. Ternary complex = presence of inactive ternary complex of TS:FdUMP:5-10 methylenetetrahydrofolate (− = no ternary complex detected, +/− = weak ternary complex signal, + = strong ternary complex signal and nd = not done). Ratios of during treatment expression to pre-treatment expression were calculated for each patient, the number of genes whose expression were increased or decreased >2-fold during treatment are shown for each patient (median = median change >2-fold, Max. = maximum fold change induced treatment.) @ = comparison of 2 pre-treatment biopsies from patient 17 demonstrated that 8 genes fell outside a +/− 2-fold range, median = 2.282, maximum = 5.284.

												Genes decreased			Genes increased		
PT	Sex	Age	Presentation stage	Resp. to chemo.	Resp. to chemoRT	Final stage	LN	Overall resp.	P53	Senesc.	Ternay complex	No.	Med.	Max.	No.	Med.	Max.
1	M	59	T3	SD	PR	T2	−	R	N	N	nd	122	0.432	0.151	42	2.236	6.059
2	F	56	T4	SD	PD	T4	−	NR	N	N	nd	137	0.411	0.164	37	2.895	9.550
3	F	40	T3	SD	SD	T3	+	NR	N	N	nd	1	0.464	0.464	74	2.453	7.376
4	M	78	Recurrent			T0	−		nd	nd	nd	85	0.399	0.134	18	3.284	11.335
5	F	53	T3	PR	SD	T1	−	R	P	N	nd	463	0.349	0.038	90	2.342	8.313
6	M	60	T4	PR	SD	T4*		R	N (N)	N	−	669	0.298	0.010	136	2.554	6.644
7	M	61	T3	CR	SD	T0	−	R	nd	nd	nd	442	0.315	0.014	161	2.387	6.400
8	M	58	T4	PD	SD	#		NR	N (N)	N	+	35	0.386	0.128	13	2.498	5.045
9	F	69	T3	PR	SD	T1	+	R	P (P)	N	+/−	164	0.363	0.085	22	2.834	11.901
10	M	64	T4	SD	SD	T3	+	NR	P (P)	N	+	275	0.349	0.047	16	2.237	4.257
11	M	30	Metastatic	PR					nd	nd	nd	149	0.382	0.067	19	2.384	11.426
12	M	63	T3	SD	PR	T3	−	R	N (N)	N	+	448	0.347	0.066	61	2.657	15.921
13	M	56	Metastatic	PR					nd	N	nd	101	0.435	0.252	205	2.965	16.415
14	F	78	T3	PR	PR	T2	−	R	nd	nd	nd						
15	M	58	Metastatic	PR					N (P)	N	+/−	499	0.332	0.066	131	2.548	26.269
16	M	44	T4	SD	PR	#		R	N (N)	N	+/−	274	0.395	0.085	28	2.756	9.763
17@	F	61	Metastatic	SD					nd	nd	nd	125	0.415	0.178	5	2.112	2.493
18	M	72	T3	SD	SD	T1	−	NR	P (P)	N	+	484	0.271	0.015	359	2.903	38.299

RNA Extraction and Microarray Analysis. mRNA was extracted, and radiolabeled single-strand cDNA was prepared and hybridized to gene arrays carrying 4132 I.M.A.G.E/LLNL cDNA clones spotted on nylon membranes (Invitrogen, Carlsbad, CA) as described previously (7). Hybridization signals were detected by phosphorimaging, and the quality of signal for each spot was confirmed by visual inspection. Poor quality spots were flagged and removed from the analysis. Signal intensity was normalized using all of the data points and a pooled reference mRNA from 3 different normal bowel samples, because synchronous normal bowel samples were not available. The yield of mRNA was sufficient for a single array run per biopsy. Therefore, a strategy of filtering the data only to include genes altered in multiple patients was used (*e.g.* Refs. 3, 8). This approach eliminates genes altered in single samples and removes genes unaffected by treatment that will not contribute to the identification of differences or similarities between patients. In this study we used a filter of a 2-fold change in gene expression in either at least 2 of 17 pretreatment profiles or in 4 or more of 34 across all of the patient samples. Unsupervised analysis, which relied on expression data alone, used a K means algorithm and a visual output generated using TreeView (8). A supervised approach was also used in which prior knowledge of response or treatment was incorporated into the data analysis used a *t* test with a two-tailed distribution and unequal variance or SAM (9). The SAM algorithm identifies genes with statistically significant changes in expression by assimilating a set of gene-specific *t* tests and assigns a score based on degree of change of gene expression relative to SD; repeated permutations of the data are then used to identify a false detection rate (9). To aid interpretation of the gene expression patterns, expression profiles of the pooled normal bowel sample, and tissue cultured colorectal cancer cells (HCT116, HT29, and SW480), T (Jurkat) and B (DOHH2) lymphocytes, endothelial cells (HUVECs), and fibroblasts were included.

Protein Analysis by Immunohistochemistry or Western Blotting. Frozen 12 μM sections were cut from a portion of the tumor biopsy and stained with H&E or with 2 μg/ml of a mixture of two antibodies that recognize wild-type and mutant conformations of p53 (NeoMarkers, Fremont, CA). Antigen-antibody complexes were detected using an APAAP system (Vector Laboratories, Burlingame, CA). Slides were also stained with a proprietary detection reagent for the senescence-associated β-galactosidase or a positive control detection solution for endogenous β-galactosidase (Trevigen Inc., Gaithersburg, MD) and counterstained with Nuclear Fast Red. In some instances, remaining tumor biopsies were analyzed by immunoblotting as described previously (7) with antibodies specific to TS (a generous gift from Dr. Wynne Aherne, Institute of Cancer Research) or p53 (NeoMarkers).

RESULTS

Treatment with MMC and Infusional 5FU Inhibits Thymidylate Synthase But Does Not Induce p53. We studied a consecutive series of tumors, biopsied by rigid sigmoidoscope, from rectal cancer patients treated with preoperative chemoradiotherapy (Table 1). Treatment was a 12-week protracted venous infusion of 5FU, a fluoropyrimidine antimetabolite (10). MMC, a bioreductive anticancer agent that induces DNA damage after enzymatic activation, was administered at the start of treatment and again at 6 weeks. This was followed by chemoradiotherapy for an additional 6 weeks with a reduced dose of infusional 5FU and CT planned radiotherapy. Pretreatment tumor biopsies were taken before the initial dose of MMC, and during-treatment biopsies were obtained 6 weeks into the primary chemotherapy treatment of a continuous infusion with 5FU, but before the second dose of MMC and before the initiation of subsequent chemoradiotherapy.

5FU is activated by metabolism and its metabolites affect multiple molecular targets by: (*a*) inhibition of TS by FdUMP; (*b*) incorporation of FdUTP into DNA; and (*c*) incorporation of FUTP into RNA (10, 11). A number of factors influence these mechanisms of action *in vivo*. The schedule of delivery is important in determining the mechanism of action, as daily or weekly pulse treatments at a high dose are thought to predominantly effect RNA metabolism, whereas a prolonged continuous infusion of a lower dose is thought to result in TS inhibition (10). 5FU has also been reported to induce p53 *in vitro* and in normal mouse intestine *in vivo* (12). Coincubation of 5FU with thymidine or uridine has demonstrated that the induction of p53 *in vivo* resulted from incorporation of FUTP into RNA (12). Induction of p53 will result in apoptosis, and/or cell cycle arrest and senescence. Because these factors would potentially influence gene expression we initially examined TS and p53 expression before expression profiling. We had sufficient material from 8 of the patients to allow immunoblot analysis of TS and p53 expression in pre- and during-treatment biopsies. An inactive ternary complex of TS:FdUMP:5–10 methylenetetrahydrofolate migrating at a slightly higher molecular weight than TS was detected in the during-treatment biopsies, but not the pretreatment samples (Fig. 1*A*; Table 1). Different levels of TS and the ternary complex were detected; however, in this small series of

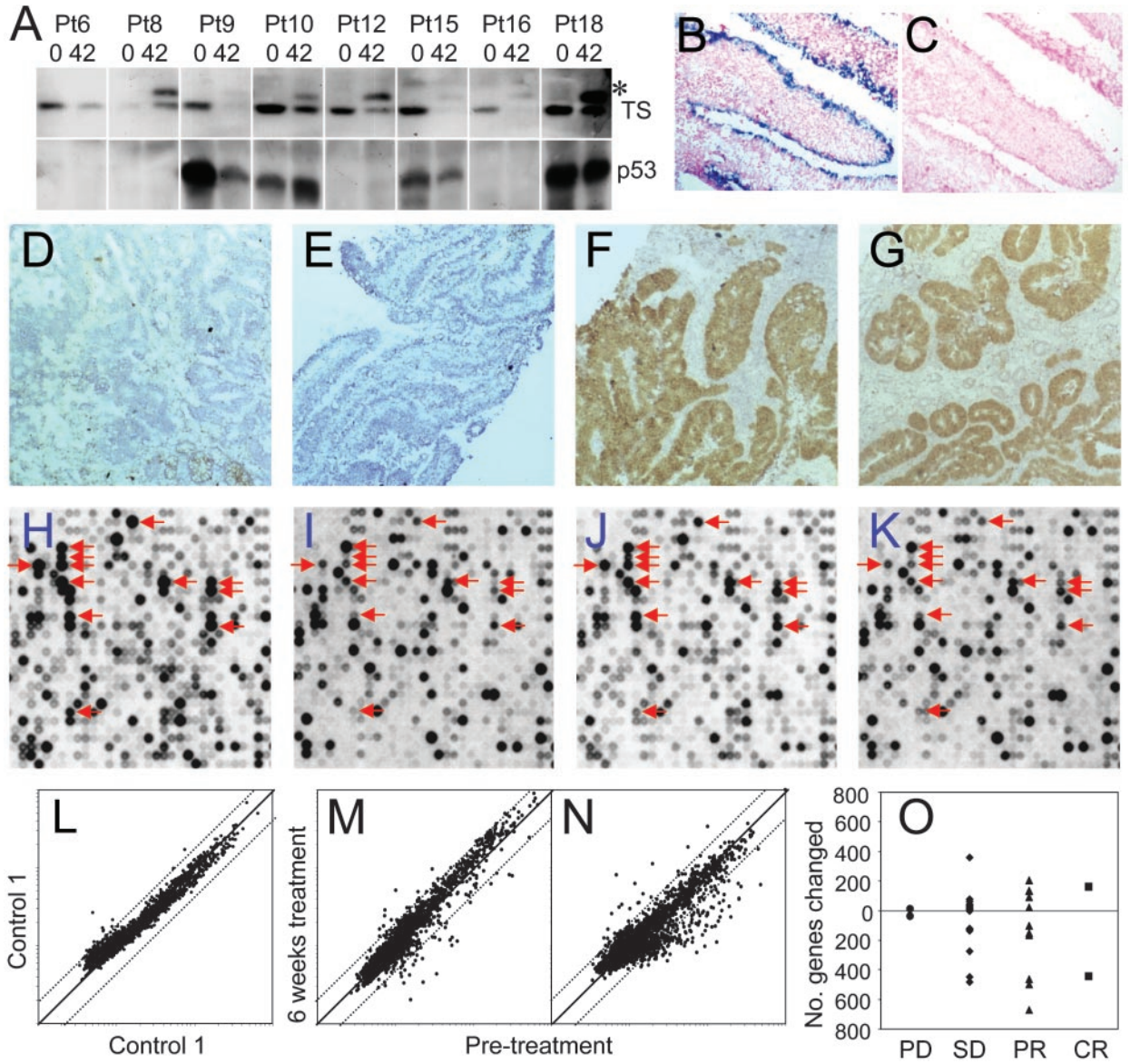

Fig. 1. *A*, Western blot analysis of TS (∗ = TS:FdUMP:5–10 methylenetetrahydrofolate ternary complex) and p53 before and during treatment. Frozen sections were stained for (*B*) β-galactosidase positive control reagent, (*C*) senescence-associated β-galactosidase (an example of a during treatment biopsy is shown), or wild-type and mutant p53 expression and counterstained with H&E before (*D* and *F*) and during (*E* and *G*) chemotherapeutic treatment of patient 6 (*D* and *E*) and patient 5 (*F* and *G*). Examples of array data from patient 6 before treatment (*H*) and during treatment (*I*), and patient 5 before treatment (*J*) and during treatment (*K*). The *arrows* indicate ribosomal protein genes. Examples of scatter plots of gene expression (arbitrary phosphorimaging units) of two separate pretreatment mRNA preparations from a single patient (*L*) or treated gene expression *versus* pretreatment gene expression from 1 patient (*M*) who progressed (patient 8) and 1 (*N*) who had a partial response (patient 7) are shown. The ——— indicates the 1:1 ratio and the ---- a 2:1 ratio. *O*, summary of genes increased or decreased after 6 weeks chemotherapy treatment (*CR*, complete response; *PR*, partial response; *SD*, stable disease; and *PD*, progressive disease).

patients there was no correlation between response or outcome and levels of TS or ternary complex (data not shown). Analysis of p53 in pre- and during-treatment samples did not reveal any consistent evidence for p53 induction during treatment (Fig. 1*A*). These observations suggested that the drug treatment regimen resulted in TS inhibition but did not induce a prolonged activation of p53. There was also no evidence for a significant increase in poly(ADP)ribose polymerase cleavage by the apoptotic protease cascade (data not shown) or for the induction of the senescence-associated β-galactosidase, after treatment (Fig. 1, *B* and *C*). In addition, the examples shown of p53-negative and p53-positive staining illustrate the general observations that (*a*) each biopsy consists mainly of tumor; (*b*) p53 staining, when detected, was restricted to tumor cells; and (*c*) consistent with the immunoblotting data there was no evidence for prolonged induction of wild-type p53 in normal tissue or tumor after treatment (Fig. 1, *D–G*; Table 1).

Chemotherapy Alters the Global Gene Expression Profile of Human Rectal Cancer Tumors. mRNA, of sufficient quality for microarray analysis, was successfully extracted from all of the biopsies. Reproducibility of the methodology was assessed by comparing 2 separate pretreatment tumor mRNA preparations from a single patient. Eight genes fell outside a ±2-fold range (median = 2.282; range 2.060–5.284) when 2 pretreatment samples from the same patient were compared (Fig. 1*L*). Analysis of pretreatment *versus* during-treatment profiles in each individual patient demonstrated evidence for altered gene expression (Fig. 1, *H–K*, *M*, and *N*). Comparison of the control *versus* control scatter plot (Fig. 1*L*) to the pretreatment *versus* during-treatment plots (Fig. 1, *M* and *N*) demonstrated clearly that both the number and degree of altered gene expression was considerably higher in the latter. The number of genes that decreased >2-fold during treatment (median of 219) generally ex-

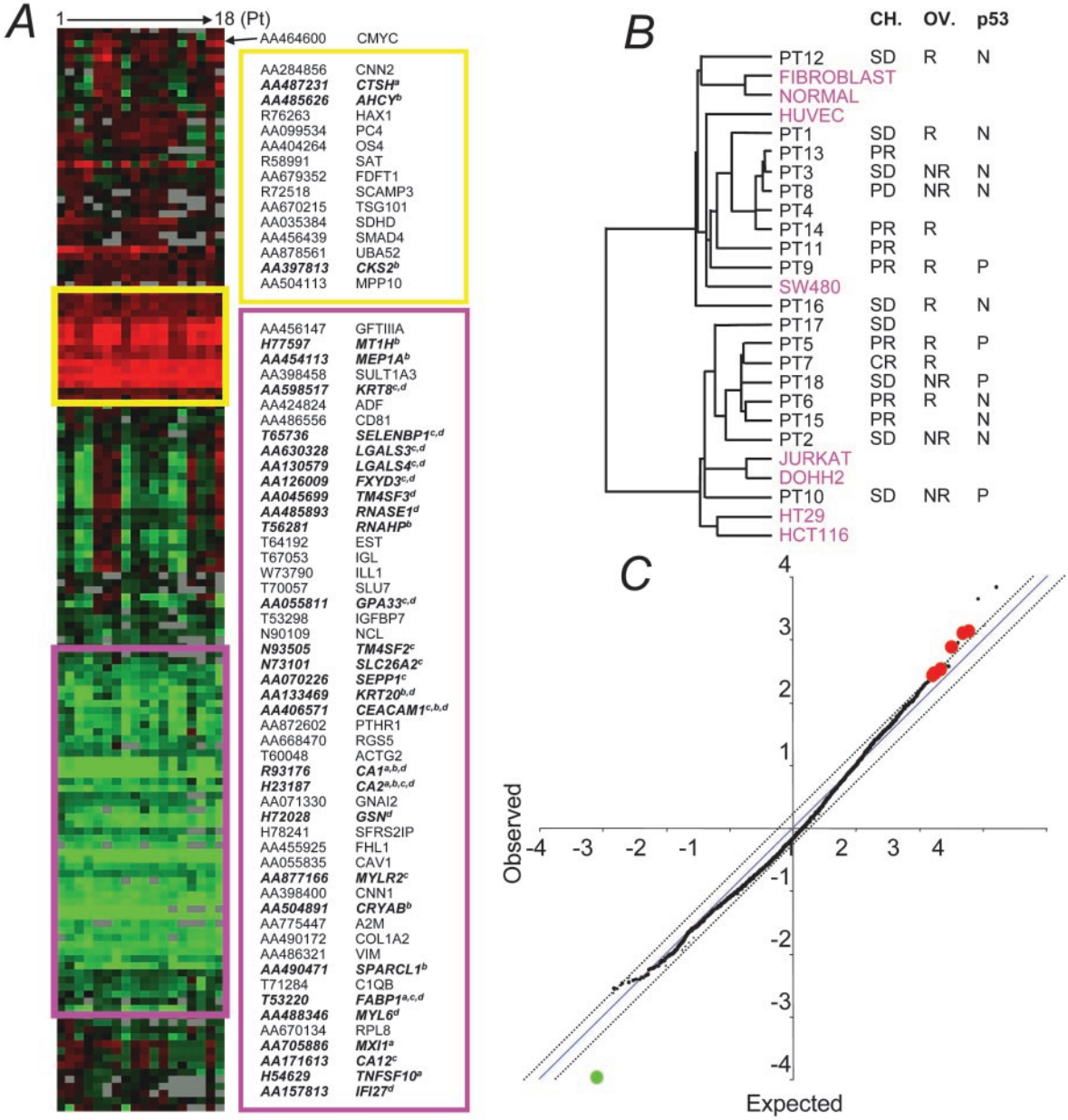

Fig. 2. Analysis of pretreatment gene expression profiles. *A*, pretreatment gene expression relative to normal bowel gene expression. The *horizontal axis* corresponds to patients 1–18 and the *vertical axis* to the tumor:normal bowel ratio for the genes listed. Data were filtered to include only genes increased by 2-fold in all pretreatment relative to the asynchronous pooled normal bowel reference RNA sample. In addition, genes reported previously to be increased or decreased in colon cancer were included for reference (13–16). Data were analyzed by K means and an output generated using TreeView (8). *Green*, decreased relative to normal bowel; *red*, increased relative to normal bowel; *black*, unchanged relative to normal bowel; *gray*, no confidence in signal. Two major clusters were detected; the *yellow box* indicates genes increased in tumor biopsies and the *pink box* genes that were decreased. *a*, Ref. 13; *b*, Ref. 14; *c*, Ref. 15; and *d*, Ref. 16. *B*, unsupervised K means analysis and TreeView output (8) of 872 genes of which the expression was altered by >2-fold from the median in at least 2 of 17 (at least 10%) pretreatment samples. Expression profiles of the pooled normal bowel sample, and tissue cultured colorectal cancer cells (*HCT116*, *HT29*, and *SW480*), T (*Jurkat*) and B (*DOHH2*) lymphocytes, endothelial cells (*HUVEC*) and fibroblasts were also included (*pink text*). *CH.*, response of the tumor to the primary chemotherapy (*CR*, complete response; *PR*, partial response; *SD*, stable disease; and *PD*, progressive disease) and *OV.*, overall response after completion of chemoradiotherapy (*R*, response; *NR*, nonresponse). *p53*, p53 immunohistochemical status (*N*, negative; *P*, positive). *C*, graphical output of observed *versus* expected score from the SAM algorithm (9). Seven genes were significantly associated with response to primary chemotherapy (complete/partial response *versus* stable/progressive disease) with a false detection rate of 0.95 genes. One gene (aldehyde dehydrogenase 7; *green spot*) was significantly higher by a factor of 2-fold in nonresponders (stable/progressive disease), and 6 genes (cytochrome b5, activating transcription factor 4, ribosomal protein S29, a vacuolar ATPase and 2 instances each of eukaryotic initiation factor 4AI, and eukaryotic translation elongation factor 1γ; *red spots*) were significantly higher by 2-fold in responders (complete/partial response).

ceeded those that increased >2-fold (median of 53; Fig. 1*O*; Table 1). However, there was no significant association between response to initial chemotherapy or overall response and the number genes changed, or the median or maximum fold-change in gene expression ($P > 0.05$; *t* test with a two-tailed distribution and unequal variance).

Analysis of Pretreatment Gene Expression by Microarray. Analysis of pretreatment gene expression relative to the pooled normal bowel reference mRNA demonstrated many similarities with studies that have previously compared expression profiles of normal and colorectal cancer tissue either using glass slide arrays or serial analysis of gene expression (Fig. 2*A*; Refs. 13–16). The normal bowel exhibited a higher level of expression of a number of classes of genes of which the products are associated with normal functions of colonocytes (*e.g. FABP1*, *FXYD3*, *SEPP1* and *SELENBP1*), hematopoietic cells (*e.g.* immunoglobulin genes *IGL* and *ILL1*), and smooth muscle (*e.g. ACTG2*, *MYLR2*, *MYL6*, and *CNN1*). Among the genes overex-

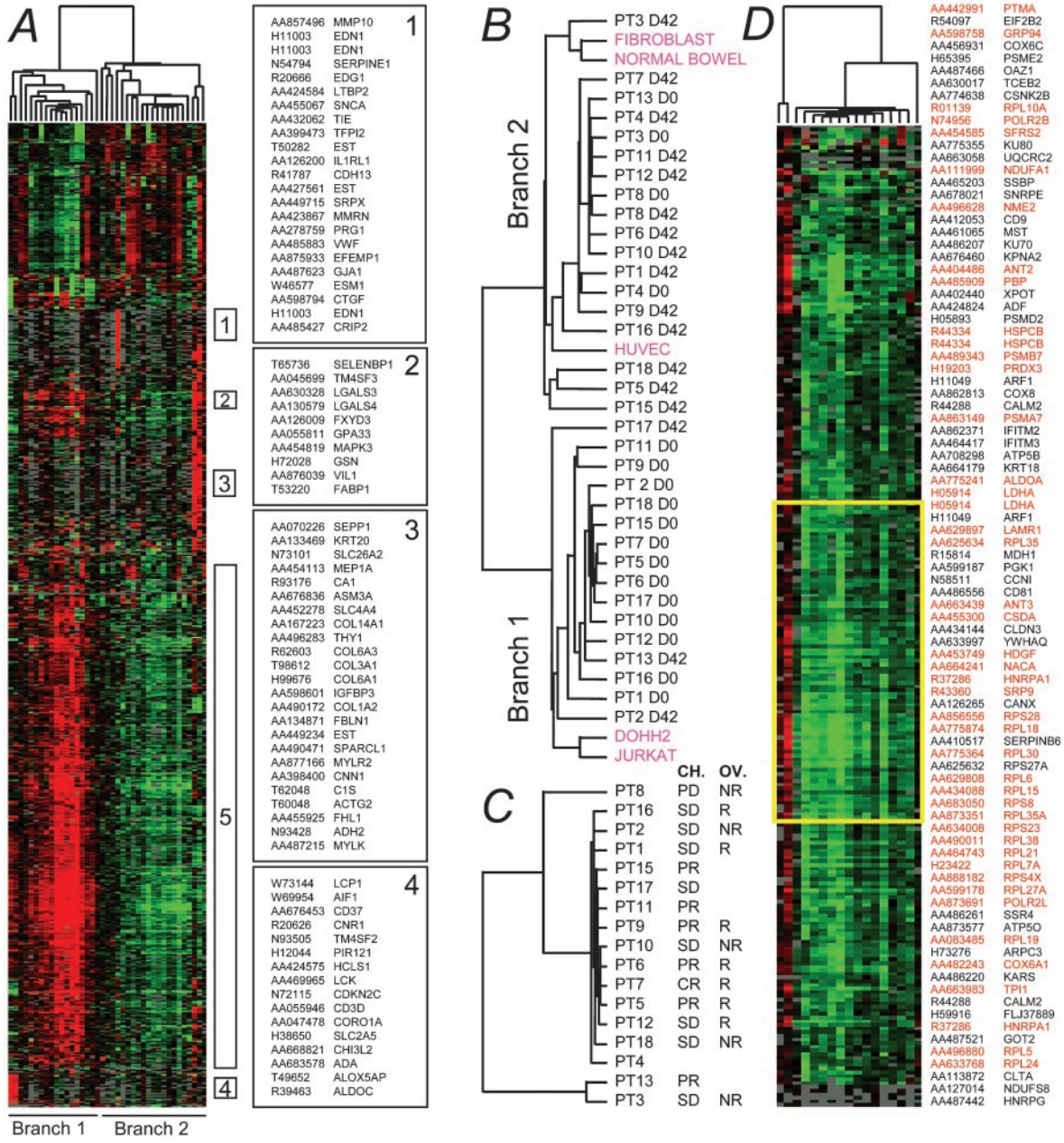

Fig. 3. *A*, unsupervised K means analysis and TreeView output (8) of 685 genes of which the expression was altered by >2-fold from the median in a minimum of 4 of 34 (≥10%) pre- and during treatment samples. *Node 1*, genes expressed in endothelial cells; *node 2*, fibroblast and normal bowel genes decreased after MMC/5FU treatment; *node 3*, genes expressed in fibroblasts and normal bowel that were not altered after MMC/5FU treatment; *node 4*, lymphocyte associated genes; *node 5*, a cluster of 338 genes decreased during MMC/5FU treatment. *Green*, decreased relative to the median; *red*, increased relative to the median; *black*, unchanged relative to the median; *gray*, no confidence in signal. *B*, dendrogram of patient samples from *A*. Expression profiles of the pooled normal bowel sample, and tissue cultured T (*Jurkat*) and B (*DOHH2*) lymphocytes, endothelial cells (*HUVEC*) and fibroblasts were also included (*pink text*). *C* and *D*, TreeView output of K means clustering of the 247 genes identified by the SAM algorithm to be significantly altered >2-fold during treatment with 5FU/MMC, plotted as a ratio of treatment:pretreatment for all patients. *CH.*, response of the tumor to the primary chemotherapy (*CR*, complete response; *PR*, partial response; *SD*, stable disease; and *PD*, progressive disease) and *OV.*, overall response after completion of chemoradiotherapy (*R*, response; *NR*, nonresponse). Genes encompassed by the *yellow box* are listed (see Table 2 and Supplemental Table 1 for full list); those demonstrated previously to be c-MYC regulated are highlighted in *red text* (19–23).

pressed in tumors compared with normal bowel were several that had been reported previously (13–16), such as the *c-MYC* oncogene, whereas *MXI1*, a repressor of c-MYC, was decreased (Fig. 2*A*).

Correlating pretreatment expression profiles with treatment outcome was not a major aim of this initial study. However, we did take the opportunity to compare the expression data from the pretreated tumor biopsies by calculating a ratio of expression relative to the median expression for each gene and analyzing these using the classical unsupervised hierarchical clustering method described by Eisen *et al.* (8). The aim was to determine whether knowledge of pretreatment expression profile alone could be used to determine the response to the primary chemotherapy or overall outcome. This approach divided the pretreatment biopsies into two major branches, but failed to separate patients with response to the initial chemotherapeutic treatment or overall outcome (Fig. 2*B*). An alternative, supervised approach was also used. In this case the data were initially separated into responders or nonresponders and then analyzed using the SAM algorithm to identify genes associated with response (9). This supervised approach comparing expression to response detected 7 genes of which the pretreatment expression levels differed by >2-fold and were significantly associated with response to chemotherapy (Fig. 2*C*). One gene, aldehyde dehydrogenase 7 (*ALDH7*), the product of which is involved in detoxi-

Table 2 *Genes identified by SAM as being significantly decreased by MMC/5FU treatment*

The dataset was divided into pre-treatment and during treatment samples from the 15 patients treated with MMC/5FU for whom we had pre- and during treatment profiles and analyzed using SAM (9). The table lists genes by general function that were significantly decreased by >2-fold during treatment. Italic text indicates genes previously reported to be c-MYC-regulated (19–23). A = genes with cDNA duplicate spots, B = genes with triplicate cDNA spots.

Protein—synthesis	
AA496880	*Ribosomal protein L5 (RPL5)*
AA629808	*Ribosomal protein L6 (RPL6)*
H23422	*Ribosomal protein L7a (RPL7A)*
T67270	*Ribosomal protein L10 (RPL10)*
R01139	*Ribosomal protein L10a (RPL10A)*
AA680244	*Ribosomal protein L11 (RPL11)*
AA434088	*Ribosomal protein L15 (RPL15)*
AA775874	*Ribosomal protein L18 (RPL18)*
AA083485	*Ribosomal protein L19 (RPL19)*
AA464743	*Ribosomal protein L21 (RPL21)*
AA633768	*Ribosomal protein L24 (RPL24)*
AA599178	*Ribosomal protein L27a (RPL27A)*
AA775364	*Ribosomal protein L30 (RPL30)*
W15277	*Ribosomal protein L31 (RPL31)*
AA625634	*Ribosomal protein L35 (RPL35)*
AA873351	*Ribosomal protein L35A (RPL35A)*
AA490011	*Ribosomal protein L38 (RPL38)*
AA888182	*Ribosomal protein S4 (RPS4X)*
T69468	*Ribosomal protein S4 (RPS4Y)*
AA683050	*Ribosomal protein S8 (RPS8)*
AA629641	*Ribosomal protein S13 (RPS13)*
AA872341	*Ribosomal protein S15a (RPS15A)*
AA66830	*Ribosomal protein S16 (RPS16)*
AA634008	*Ribosomal protein S23 (RPS23)*
AA625632	Ribosomal protein S27a (RPS27A)
AA856556	*Ribosomal protein S28 (RPS28)*
AA411343	Ribosomal protein S29 (RPS29)
R51607	Translation initiation factor (EIF1)
R93621	Eukaryotic translation initiation factor 2 sub. 2 (EIF2S2)
R54097	Eukaryotic translation initiation factor 2 sub. 2 (EIF2S2)[A]
AA668703	Eukaryotic translation initiation factor 3 sub. 4 (EIF3S4)
AA669674	Eukaryotic translation initiation factor 3 sub. 6 (EIF3S6)
AA598863	*Eukaryotic translation initiation fact. 3 sub. 8 (EIF3S8)*
H09590	*Eukaryotic translation initiation factor 4A1 (EIF4A1)*[A]
R43973	*Eukaryotic translation elongation factor 1 γ(EEF1G)*[A]
R43766	*Eukaryotic translation elongation factor 2 (EEF2)*
AA629909	Glycyl-tRNA synthetase (GARS)
AA410636	*Isoleucine-tRNA synthetase (IARS)*
AA486220	Lysyl-tRNA synthetase (KARS)
AA634166	Glutaminyl-tRNA synthetase (QARS)
RNA processing	
R37286	*H'geneous nuclear ribonucleoprotein A1 (HNRPA1)*[A]
AA126911	*H'geneous nuclear ribonucleoprotein A1 (HNRPA1)*
W72693	H'geneous nuclear ribonucleoprotein A, B (HNRPAB)
W02101	H'geneous nuclear ribonucleoprotein A2, B1 (HNRPA2B1)
H05899	H'geneous nuclear ribonucleoprotein C1, C2 (HNRPC)[A]
AA487442	Heterogeneous nuclear ribonucleoprotein G (HNRPG)
AA599116	Small nuclear ribonucleoprotein B, B1 (SNRPB)
T62529	Small nuclear ribonucleoprotein D2 (SNRPD2)
AA678021	Small nuclear ribonucleoprotein E (SNRPE)
AA668189	Small nuclear ribonucleoprotein F (SNRPF)
AA133577	*Small nuclear ribonucleoprotein G (SNRPG)*
AA454585	*Splicing factor arginine, serine-rich 2 (SFRS2)*
AA663986	*Fibrillarin (FBL)*
AA476294	*Nucleolin (NCL)*
AA894577	*Nucleolar protein (NOP56)*
AA402440	Exportin (XPOT)
H29485	La autoantigen (SSB)
AA490047	Poly(rC)-binding protein 1 (PCBP1)
W72816	3′ pre-RNA cleavage stimulation factor sub. 1 (CSTF1)
Metabolism—oxidative phosphorylation	
AA708298	ATP synthase H+ transporting, mitochon. F1 β (ATP5B)
AA644234	ATP synthase H+ transporting, mitochon. F1γ1 (ATP5C1)
AA453765	ATP synthase H+ transporting mitochon. F0 β1 (ATP5F1)
AA431433	ATP synthase H+ transporting, mitochon. F0 ϵ (ATP5I)
AA873577	ATP synthase H+ transporting mitochon. F1 O (ATP5O)
AA482243	*Cytochrome c oxidase subunit VIA 1 (COX6A1)*
Protein—turnover	
AA047338	*Proteasome alpha subunit type 6 (PSMA6)*
AA863149	*Proteasome alpha subunit type 7 (PSMA7)*
AA489343	*Proteasome subunit beta type 7 (PSMB7)*
T68758	Proteasome subunit beta type 1 (PSMB1)
N53065	Proteasome subunit beta type 2 (PSMB2)
AA864479	Proteasome subunit beta type 5 (PSMB5)
H05893	Proteasome 26S subunit non-ATPase 2 (PSMD2)
T47815	Proteasome activator subunit 1 (PSME1)
H65395	Proteasome activator subunit 2 (PSME2)
AA430504	UbiquitincarrierproteinE2-C(UBCH10)
N33920	Ubiquitin D(UBD)
AA676484	Calpain, small subunit (CAPNS1)
H22919	Cystatin B (CSTB)
AA486275	Proteinase inhibitor clade B member 1 (SERPINB1)
AA410517	Proteinase inhibitor clade B member 6 (SERPINB6)
AA845156	Serine protease inhibitor, Kazal type 1 (SPINK1)
Protein—folding	
AA448396	*Heat shock 10kD protein 1 (HSPE1)*
AA629567	*Heat shock cognate 71 (HSPA8)*
R44334	*Heat shock 90 beta (HSPCB)*[A]
AA411202	Activator of Hsp90 ATPase (AHA1)
AA669341	p23 co-chaperone (P23)
R60933	*Chaperonin containing TCP1 gamma subunit 3 (CCT3)*
AA598637	Chaperonin containing TCP1 subunit 4 (CCT4)
AA87269	Chaperonin containing TCP1 subunit 6A (CCT6A)
H07880	Chaperonin containing TCP1 subunit 6A (CCT6A)[A]
R43360	*Signal recognition particle 9kD (SRP9)*
AA486261	Signal sequence receptor delta (SSR4)
AA664241	*Nascent-polypeptide-associated complex alpha (NACA)*
AA598758	*Tumor rejection antigen 1 (GRP94)*
R78585	Calumenin (CALU)
AA126265	Calnexin (CANX)
AA625981	FK506-binding protein 1A (FKBP1A)
RNA transcription	
H20856	Basic transcription factor 2 (BTF2)
R83000	Basic transcription factor 3 (BTF3)
AA115186	General transcription factor II-I (GTF2I)
N74956	*RNA polymerase II polypeptide B (POLR2B)*
AA873691	*RNA polymerase II polypeptide L (POLR2L)*
AA136533	Transcription elongation factor B peptide 1-like (TCEB1L)
AA630017	Transcription elongation factor B polypeptide 2 (TCEB2)
AA099534	Activated RNA pol. II transcription cofactor 4 (PC4)
AA600217	Activating transcription factor 4 (ATF4)
AA455300	*Cold shock domain associated (CSDA)*
AA112660	Forkhead box F1 (FKHL5)
AA894687	Interleukin-enhancer binding factor 2 (ILF2)
AA664389	Transforming growth factor beta-stimulated ptn (TSC22)
AA599175	*Y-box binding protein 1 (YBP1)*
AA773894	Zinc finger protein 43 (HTF6)
Metabolism—glycolysis	
AA775241	*Aldolase A (ALDOA)*
H16958	*Glyceraldehyde-3-phosphate dehydrogenase (GAPD)*[A]
H05914	*Lactate dehydrogenase A (LDHA)*[A]
AA599187	Phosphoglycerate kinase 1 (PGK1)
AA663983	Triosephosphate isomerase 1 (TPI1)
Metabolism—other	
T59245	Methionine adenosyltransferase II alpha (MAT2A)
AA487466	Ornithine decarboxylase antizyme 1 (OAZ1)
R58991	Spermidine, spermine N1-acetyltransferase (SAT)[A]
AA679907	Isocitrate dehydrogenase 2 (IDH2)
AA487521	Mitochon. glutamic-oxaloacetic transminase 2 (GOT2)
R15814	Malate dehydrogenase 1 (MDH1)[A]
AA676223	Acyl-Coenzyme A oxidase 3 pristanoyl (ACOX3)
T65790	Farnesyl diphosphate synthase (FDPS)
R43558	Mitochondrial enoyl Coenzyme A hydratase (ECHS1)
N71160	Cytochrome c oxidase subunit VIb (COX6B)
AA456931	Cytochrome c oxidase subunit VIC (COX6C)
AA862813	Cytochrome c oxidase subunit VIII (COX8)
R53311	*Cytochrome c (HCS)*
AA111999	*NADH dehydrogenase 1 alpha 1 (NDUFA1)*
AA680322	NADH dehydrogenase 1 alpha subcomplex 4 (NDUF4)
AA127014	NADH dehydrogenase Fe-S protein 8 (NDUFS8)
AA922326	NADH dehydrogenase flavoprotein 2 (NDUFV2)
AA663058	Ubiquinol-cytochrome c reductase core ptn II (UQCRC2)
R12802	Ubiquinol-cytochrome c reductase core ptn II (UQCRC2)
AA404486	*Adenine nucleotide translocator 2 (ANT2)*
AA663439	*Adenine nucleotide translocator 3 (ANT3)*
R61295	*Adenine nucleotide translocator 3 (ANT3)*[A]
AA044059	*Voltage-dependent anion channel 1 (VDAC1)*
AA486200	Mitochondrial phosphate carrier (PHC)
Signal transduction	
R37953	Adenylyl cyclase-associated protein (CAP)
AA863086	Adenosine A3 receptor (ADORA3)
AA857163	Amphiregulin (AREG)
AA679177	Butyrate-induced transcript 1 (HSPC112)
N21624	*14-3-3 epsilon (YWHAE)*
AA633997	14-3-3 tau (YWHAQ)
AA464731	Calgizzarin (S100A11)
R44288	Calmodulin 2 (CALM2)[A]
AA774638	Casein kinase 2 beta (CSNK2B)
AA451935	Fibroblast growth factor 2-interacting factor (AP15L1)
R43581	Guanine nucleotide binding α stimulating 1 (GNAS1)[A]
R96220	*Guanine nucleotide binding β2-like 1 (GNB2L1)*

Table 2 *Continued*

H05619	GDNF family receptor alpha 2 (GFRA2)
AA453749	*Hepatoma-derived growth factor (HDGF)*
T57556	*Histidine triad nucleotide-binding protein (HINT)*
AA157813	Interferon, alpha-inducible protein 27 (IFI27)
AA419251	Interferon induced transmembrane protein 1 (IFITM1)
AA862371	Interferon induced transmembrane protein 2 (IFITM2)
AA464417	Interferon induced transmembrane protein 3 (IFITM3)
AA412053	p24 transmembrane 4 superfamily (CD9)
AA485909	*Prostatic binding protein (PBP)*
N33955	Protein phosphatase ID (PM1D)
AA406362	Prostaglandin E receptor 3 (PTGER3)
W47485	*Sigma receptor (SR-BP1)*
AA045699	Transmembrane 4 superfamily member 3 (TM4SF3)
Transport	
R49530	Accessory proteins BAP31, BAP29 (BAP31)[A]
AA625628	Accessory proteins BAP31, BAP29 (BAP31)
H11049	ADP-ribosylation factor 1 (ARF1)[A]
T71316	ADP-ribosylation factor 4 (ARF4)
AA873355	ATPase, Na+, K+ transporting alpha 1 (ATP1A1)
AA113872	Clathrin light polypeptide (CLTA)
N20335	Clathrin, light polypeptide (CLTB)
AA485913	Chloride intracellular channel 1 (CLIC1)
AA676460	Karyopherin alpha 2 (KPNA2)
T70031	Neutral amino acid transporter 5 (SLC1A5)
AA464528	Proteolipid protein 2 (PLP2)
AA444051	S100 calcium-binding protein A10 (S100A10)
AA775415	*Sentrin 2 (SMT3H2)*
AA664077	Vacuolar ATPase (ATP6S14)
Miscellaneous and unknown function	
AA404264	Amplified in osteosarcoma (OS4)
AA457092	Chromosome 20 open reading frame 47 (C20ORF47)
R51835	EST[A]
R60313	EST[B]
AA702548	*Etoposide-induced mRNA (PIG8)*
R42479	FLJ20528
H59916	FLJ37889
AA460728	FLJ38479
AA082943	Hmd4a12
N98412	Lysozyme (LYZ)
AA608575	Propionyl Coenzyme A carboxylase α polypep. (PCCA)
AA676466	*Argininosuccinate synthetase (ASS)*
AA644092	*Expressed in non-metastatic cells 1 (NME1)*
AA496628	*Expressed in non-metastatic cells 2 (NME2)*
AA459909	Dimerization cofactor of hepatocyte factor 1α (PCBD)
H59231	*Meltrin gamma (ADAM9)*
AA463497	Membrane cofactor protein (MCP)
AA683321	Prader-Willi, Angelman region 5 (PAR5)
W92812	Pro-platelet basic protein (PPBP)
AA457719	Reticulocalbin 1 (RCN1)
AA447684	Small proline-rich protein 1B (SPRR1B)
T65736	Selenium binding protein 1 (SELENBP1)
AA400474	Zona pellucida binding protein (ZPBP)
Detoxification	
AA136710	Glyoxalase I (GLO1)
AA135152	Glutathione peroxidase 2 (GPX2)
AA495936	*Microsomal glutathione S-transferase 1 (MGST1)*
W73474	Microsomal glutathione S-transferase 2 (MGST2)
AA775803	Peroxiredoxin 1 (PRDX1)
H68845	*Peroxiredoxin 2 (PRDX2)*
H19203	*Peroxiredoxin 3 (PRDX3)*
AA459663	*Peroxiredoxin 4 (PRDX4)*
T64482	S-formylglutathione hydrolase (ESD)
AA599127	Superoxide dismutase 1 (SOD1)
AA461065	Mercaptopyruvate sulfurtransferase (MST)
Chromatin and DNA synthesis	
AA486207	70kD Ku antigen (KU70)
AA775355	80kD Ku antigen (KU80)
AA608557	Damage-specific DNA binding protein 1 (DDB1)
AA683085	High-mobility non-histone chromosomal ptn 1 (HMG1)
AA608514	*H3 histone 3B (H3,3B)*
AA668811	H3 histone 3A (H3F3A)
AA127093	Histone deacetylase 3 (HDAC3)
AA465203	Mitochon. single-stranded DNA-binding protein (SSBP)
AA450265	*Proliferating cell nuclear antigen (PCNA)*
AA608548	*SET translocation (SET)*
Cell cycle and proliferation	
N58511	Cyclin I (CCNI)
AA488332	Proliferation-associated 2G4 (PA2G4)
AA442991	*Prothymosin alpha (PTMA)*
R32848	S100 calcium-binding protein P (S100P)
Cytoskeleton	
AA634006	Actin alpha 2 (ACTA2)
R44290	Actin beta (ACTB)[A]
R42609	Actin filament capping protein alpha 2 (CAPZA2)
R40850	Centractin alpha (ACTR1A)[A]
H73276	Actin related protein 2, 3 complex subunit 3 (ARPC3)
T65118	Catenin alpha 1 (CTNNA1)[A]
AA434144	Claudin 3 (CLDN3)
AA644679	Cytoplasmic dynein light polypeptide (PIN)
AA424824	destrin (ADF)
AA664179	Keratin 18 (KRT18)
AA634103	Thymosin beta 4 (TMSB4X)
AA888148	Tubulin beta 2 (TUBB2)
Cell surface and adhesion	
AA054073	Carcinoembryonic antigen-related cell adhesion 6 (NCA)
AA130579	Galectin 4 (LGALS4)
AA485353	Galectin 6 binding protein (LGALS3BP)
AA629897	*Laminin receptor 1 (LAMR1)*
AA458965	Natural killer cell transcript 4 (NK4)
AA486556	Target of antiproliferative antibody 1 (CD81)
Immune	
AA670408	Beta-2-microglobulin (B2M)
AA663981	Immunoglobulin heavy constant gamma 3 (IGHG3)
H54023	Ig-like receptor subfamily B member 2 (LILRB2)
AA464246	Major histocompatibility complex, class I, C (HLA-C)
AA702254	Major histocompatibility class II DN alpha (HLA-DNA)
R47979	Major histocompatibility class II DR alpha (HLA-DRA)
AA634028	Major histocompatibility class II DP alpha (HLA-DPA)

fication, particularly lipid peroxidation, was higher in nonresponders. Six genes (*EEF1G, EIF4A1, CYB5-M, ATF4, RPS29,* and *ATP6S14*) were lower in responders. There was no obvious mechanistic link to treatment for any of these genes, although expression of *EEF1G* has been reported previously to be increased in gastrointestinal cancers (17, 18). However, follow-up studies in a larger cohort of patients will be required to validate the significance of these potential markers.

Gene Expression Profiling Detects Altered Gene Expression After Chemotherapy. The principle objective of the study was to assess the feasibility of microarray profiling to investigate gene expression changes induced by therapy in the tumor tissue of cancer patients. Fig. 3 shows that the expression profiles from the pre- and during-treatment biopsies were divided between two major branches after unsupervised hierarchical clustering. Branch 1 (Fig. 3*A*, left side, and Fig. 3*B*, bottom) contained 13 pretreatment samples, 3 treatment biopsies, and also the B (DOHH2) and T (Jurkat) cell lines that were included for comparison. Branch 2 (Fig. 3*A*, right side, and Fig. 3*B*, top) contained 14 during treatment samples, 4 pretreatment biopsies, normal bowel, endothelial (HUVEC) cells, and fibroblasts. Clusters of genes of which the products were associated with lymphocytes, stroma, muscle, or normal bowel were identified (Nodes 1–4, respectively, Fig. 3*A*). With one exception, a node with some genes associated with normal colon function, there was no major difference in the distribution of the genes associated with normal tissue types between the pre- and during-treatment tumor expression profiles for each patient. This indicated that any differences in gene expression profile during treatment were unlikely to result from altered content of normal tissue types in the tumor biopsies. The exception was the expression of a subset of genes we had shown to be enriched in the normal bowel pool that were decreased in branch 2 (predominantly during treatment samples; Fig. 2*A*; Node 2, Fig. 3*A*).

Of particular interest was a large node containing 338 genes that also had lower expression in the during treatment branch 2 (Node 5, Fig. 3*A*). Within this node 297 genes exhibited significantly different expression between branch 1 and branch 2 ($P < 0.05$; t test with a two-tailed distribution and unequal variance). These included 40 genes encoding products involved in RNA synthesis and processing, 79 genes involved in protein synthesis and processing, and 53 genes involved in metabolism, particularly ATP production. Interestingly, 90 of the 297 genes in this "synthesis and metabolism" gene cluster had been reported previously in expression profiling studies as having increased expression in response to c-MYC expression (19–22). The array experiments detected a 1.8-fold median decrease (range, 0.8–4.5) in c-MYC expression in patient samples during treatment, and this was confirmed by real-time PCR of remaining RNA from 2 patients (data not shown).

The dataset was then divided into pretreatment and during-treatment samples from the 15 patients treated with MMC/5FU for whom we had pre- and during-treatment profiles, and analyzed using the SAM algorithm (9). This analysis did not reveal any genes with significantly increased expression during treatment, but did identify a set of genes that exhibited significantly decreased expression during treatment that were encompassed by the "synthesis and metabolism" cluster described above (Fig. 3*A*; Table 2). In this case 247 individual genes enriched with those encoding products involved in RNA synthesis (33), protein synthesis (80), and metabolism (38) showed significantly decreased expression by >2-fold during treatment; of these 80 had been reported previously as c-MYC regulated genes (Table 2; Refs. 19–22). These also included genes encoding the molecular chaperones *HSP90β* and *GRP94*, and also a number of their cochaperones that stimulate chaperone activity (*p23*, *AHA1*, *CDC37*, and *HSC71*; Table 2). The ratios of treatment to pretreatment expression levels for these 247 genes across all of the patients were also analyzed by hierarchical clustering (Fig. 3, *C* and *D*). Thirteen of the 15 patients treated with MMC/5FU clustered closely together. The decrease in expression of the "synthesis and metabolism" genes, although detected, was not as pronounced in patient 8, the only patient that exhibited progressive disease during this study. Two patients (3 and 13) gave a different expression pattern in response to treatment (Fig. 3*C*, bottom, and Fig. 3*D*, left). Although care has to be taken, as these were single expression profiles, possible explanations for their different gene signatures were considered. Patient 13 was treated with irinotecan, a topoisomerase I inhibitor, suggesting that a difference in treatment regime may be responsible for the different expression profile. Patient 3 was treated with MMC/5FU, but interestingly had lower pretreatment levels of c-MYC compared with the other patients, which may account for the different profile (Fig. 2*A*). Also of note, the single patient treated with the specific TS inhibitor raltitrexed (patient 17) gave a clustering pattern similar to patients treated with MMC/5FU, consistent with a view that this altered gene expression pattern may be associated with TS inhibition.

DISCUSSION

In this study we set out to assess whether it was feasible to use gene expression profiling by cDNA microarray to detect altered gene expression in solid tumor tissue in response to chemotherapy treatment of patients with rectal cancer. Various studies have been carried out previously to profile changes in gene expression in cultured cancer cell lines treated with established or developmental therapeutic agents (reviewed in Ref. 1), but to our knowledge this is the first study to attempt to do this in a clinical setting. Importantly, we were able to obtain material of sufficient quantity and quality to successfully profile gene expression by microarray. The primary purpose of the study was to determine changes in global gene expression that occurred during drug treatment. Altered gene expression patterns were apparent in all of the patients after treatment. The inclusion of expression profiles from different cell types suggested that these changes did not arise from altered normal tissue content. However, a subset of genes that were expressed in normal bowel was decreased in the tumor samples by treatment. These changes may have resulted from effects of MMC/5FU on normal cell types. In this study we did not have access to normal bowel samples pre- and during treatment. However, in future studies it would be useful to include such samples, where this is clinically feasible, to determine the effects of treatment on normal tissue.

Interestingly, a major "synthesis and metabolism" cluster of genes was detected in the tumor samples, the expression of which was decreased during treatment. Two thirds of these genes encoded products involved in RNA, and protein synthesis and processing, and also many involved in cellular metabolism, particularly ATP production. Notably, a third of these genes had been reported in previous expression mRNA and protein profiling studies as being positively regulated by c-MYC (19–23). Consistent with this observation decreased c-MYC expression was also observed and validated. There was also some overlap between the "synthesis and metabolism" cluster that we describe here to be down-regulated by the chemotherapy regimen and genes reported to be decreased in expression after differentiation of Caco-2 cells, a process associated with a cell cycle arrest and decreased proliferation (24). This observation would be consistent with our "synthesis and metabolism" cluster potentially being a proliferation-regulated set of genes. However, Mariadason *et al.* (24) also reported increased expression of genes encoding ribosomal protein genes during differentiation. This was in contrast with the decreased expression of ribosomal protein genes that we detected here, and that have been reported in mRNA and protein expression profiling of c-MYC-regulated genes (19–23). Inhibition of c-MYC expression or activity results in reduced proliferation of tumor cells (25–28). Therefore, the expression pattern that we detected could reflect: (*a*) MMC/5FU treatment inhibiting the activity or expression of c-MYC, which then subsequently results in reduced proliferation, or alternatively; b) the expression pattern may be a direct result of reduced proliferation or loss of cells from the S phase of the cell cycle that leads to reduced c-MYC expression or activity; or (*c*) some combination of the two. Treatment of colon cancer cell lines with 5FU *in vitro* results in an accumulation of cells in early S phase (data not shown); however, it is unclear from the literature whether the same occurs in tumor cells after 5FU treatment of patients. The expression profiling did not reveal altered expression of genes encoding products that regulate cell cycle progression or are expressed in a particular phase of the cell cycle that might indicate accumulation or exit of cells from a particular phase of the cell cycle. Therefore, future experiments, including expression profiling *in vitro* after treatment with c-MYC interfering RNA or peptide inhibitors of c-MYC, are now underway to examine and distinguish between the influence of c-MYC and proliferation on the expression of genes in the "synthesis and metabolism" cluster that we have identified.

Interestingly, the single patient treated with the specific TS inhibitor raltitrexed, but not the patient treated with the topoisomerase I inhibitor irinotecan, clustered with the MMC/5FU-treated patients. Clearly care has to be taken in the interpretation of the data from these single patients. However, it is not unreasonable to speculate that the clustering pattern suggests that the gene expression changes seen with 5FU treatment may represent a downstream consequence of TS-inhibition resulting in reduced MYC activity. Because the irinotecan-treated patient was responsive to the drug, it can be tentatively concluded that the gene expression signature observed in during MMC/5FU treatment might not be a consequence of a general antiproliferative effect. Also, consistent with the gene expression profile being associated with TS inhibition, we detected the inactive ternary complex of TS:FdUMP:5–10 methylenetetrahydrofolate in tumor tissue during MMC/5FU treatment.

The nature of the mechanistic link between MMC/5FU treatment and c-MYC *in vivo* is not clear. However, there is evidence that patients with colorectal cancers that have amplified c-MYC have significantly improved outcome after adjuvant treatment with 5FU/levamisole (29). It is also of interest to note a recent observation that brief inactivation of c-MYC is sufficient to induce sustained tumor regression in a c-MYC driven *in vivo* tumor model (30). This raises the interesting possibility that decreased activity or expression of c-MYC or c-MYC-regulated genes could participate directly in the antitumor mechanism of action of MMC/5FU treatment *in vivo*.

To our knowledge this is the first clinical study to successfully profile global gene expression specifically in response to chemotherapy by comparing patient biopsies taken before and during drug exposure. The major objective of this relatively small study was to demonstrate the feasibility of the approach and in addition to obtain an initial characterization of the changes in gene expression associated with the chemotherapy regimen. Both of these objectives were

achieved. The effects were detected irrespective of response, suggesting that they were not a consequence of treatment outcome, but were more likely a consequence of drug action at its target or a secondary downstream event after target modulation. Interestingly, although we detected evidence for TS inhibition in the tumor tissue, a mechanism consistent with the infusional route of 5FU administration (10), we also detected decreased expression of genes required for RNA synthesis and processing. This suggests that infusional 5FU could exert effects on RNA metabolism by a mechanism distinct from the RNA-dependent effects caused by the incorporation of 5FUTP into RNA (10–12). Importantly, expression profiling facilitated the novel discovery that 5FU treatment caused down-regulation of genes described previously as being c-MYC-regulated. Thus, the treatment regimen was reverting transcriptional changes associated with expression of a known oncogene. This effect was unlikely to have been detected by conventional approaches and illustrated that the hypothesis-generating power of gene expression profiling may be valuable in the clinical setting, as well as in preclinical studies. Clearly, the observed changes need to be followed up in prospective studies with larger numbers of patients where the relationship with response could also be addressed in greater detail.

In addition to suggesting potential important molecular effects of 5FU, our array profiling study also points to a possible new combination treatment for colorectal cancer. In particular, the down-regulation of the molecular chaperone HSP90 in the tumor biopsies by 5FU, and in addition the reduced expression of various cochaperones, such as the HSP90 activating protein AHA1 (31) suggests that the combination of 5FU with an HSP90 inhibitor such as 17-allyamino-, 17-demethoxygeldanamycin (currently undergoing clinical trials; Ref. 7) could be effective. Additional studies are required to investigate this interesting possibility.

Gene expression profiling studies of the present type have to accommodate logistical and ethical considerations, particularly those associated with the need for repeat tumor biopsy. However, our initial observations have demonstrated both the feasibility and utility of gene expression profiling as a valuable hypothesis-generating tool for studying the molecular pharmacology of cancer drugs in the clinical setting. With judicious study design, such as the inclusion of samples to examine effects of treatment on gene expression in normal tissue and of earlier time points to detect more transient responses to drug exposure, global expression profiling has the potential to enhance the development of both established and novel anticancer agents, and also to facilitate the understanding of their mode of action in the clinic.

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