Molecular Pharmacology of Cancer Therapy in Human Colorectal Cancer by Gene Expression Profiling

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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 therapies 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 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 therapies 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 response to 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 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/m²/day) was delivered via a Hickman line for 12 weeks. MMC (7 mg/m²) 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/m²/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 performed using MRI scans according to WHO criteria (6). The radiologists were unaware of the microarray data but were aware that the patients were undergoing chemoradiotherapy. 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/m²; patient 13) or raltitrexed (5.1 mg/m²; patient 17) at a single dose every 3 weeks, were also included.

The abbreviations used are: MMC, mitomycin c; 5FU, 5-fluorouracil; SAM, significant analysis of microarrays; TS, thymidylate synthetase; MRI, magnetic resonance imaging; CT, computed tomography; HUVEC, human umbilical vascular endothelial cell; FuUMP, 5-fluoro-UMP; HSP, heat shock protein; FuUTP, 5-fluoro-UTP; FUTP, 5-fluoro-UTP.
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 assigning 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, endo- 

Table 1 Patient characteristics and response to treatment

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<th>Resp. to chemorT</th>
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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 sigmoidoscopy, 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 chemoradiotherapy 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 byFdUMP; (b) incorporation ofFdUTP into DNA; and (c) incorporation ofFUTP 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 vitro resulted from incorporation of FUTP into RNA (12). Induction ofp53 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'-methyltetrahydrofolate migrating at a slightly higher molecular weight than TS was detected in the during-treatment biopsies, but not the pretreatment samples (Fig. 1A; Table 1). Different levels of TS and the ternary complex were detected; however, in this small series of
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. 1A). 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 /H9252-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. 1L). 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. 1L) 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-
ceeded those that increased >2-fold (median of 53; Fig. 1O; Table 1). However, there was no significant association between response to initial chemotherapy or overall response and the number of 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 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, pretreatment gene expression relative to normal bowel expression (Fig. 2; 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. $FABP1$, $SEPP1$ and $SELENBP1$), hematopoietic cells (e.g. immunoglobulin genes $IGL$), and smooth muscle (e.g. $ACTG2$, $MYL2$, $MYL6$, and $CNN1$). Among the genes overexpression

Fig. 2. Analysis of pretreatment gene expression profiles. A, pretreatment gene expression relative to normal bowel 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.

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 $SW480$) were also included (pink box). 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 1α; red spots) were significantly higher by 2-fold in responders (complete/partial response).

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. 2A; 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. $ACTG2$, $MYL2$, $MYL6$, and $CNN1$). Among the genes over-expressed...
pressed in tumors compared with normal bowel were several that had been reported previously (13–16), such as the c-MYC oncogene, whereas MXII, a repressor of c-MYC, was decreased (Fig. 2A).

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 chemotherapy treatment or overall outcome (Fig. 2B). 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. 2C). 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.

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Protein—folding

AA484596 Heat shock 10kD protein 1 (HSP1)
AA629567 Heat shock cognate 71 (HSP71)
AA451344 Heat shock 90 beta (HSP90B1)
AA411202 Activator of Hsp90 ATPase (AHA1)
AA669341 p23 co-chaperone (P23)
AA96933 Chaperonin containing TCP1 gamma subunit 3 (CCT3)
AA98637 Chaperonin containing TCP1 subunit 4 (CCT4)
AA87269 Chaperonin containing TCP1 subunit 6A (CCT6A)
R07880 Chaperonin containing TCP1 subunit 6A (CCT6A)
AA41360 Signal recognition particle 9D (SRP9)
AA484621 Signal sequence receptor delta (SSR4)
AA664241 Nascent-polypeptide-associated complex alpha (NACA)
AA398738 Tumor rejection antigen 1 (GRF94)
R78558 Calumenin (CALU)
AA126265 Calnexin (CANX)

RNA transcription

H26056 Basic transcription factor 2 (TFB2)
R83000 Basic transcription factor 3 (TFB3)
AA115186 General transcription factor II (GTF2I)
AA79596 RNA polymerase II polypeptide L (POLR2L)
AA879091 RNA polymerase II polypeptide L (POLR2L)
AA136533 Transcription elongation factor beta peptide 1-like (TCEB1L)
AA360017 Transcription elongation factor 2 polypeptide 2 (TF2)
AA999534 Activated RNA pol. II transcription cofactor 4 (PC4)
AA800027 Activating transcription factor 6 (ATF6)
AA455300 Cold shock domain associated (CSDA)
AA112660 Forkhead box F1 (FKHL5)
AA988948 Interleukin-enhancing factor 2 (ILF2)
AA463489 Transforming growth factor beta-stimulated ptm (TSC2)
AA599175 Y-box binding protein 1 (YBP1)
AA773894 Zinc finger protein 43 (HTFP)

Metabolism—glycolysis

AA775241 Aldolase A (ALDOA)
H16058 Glyceroldehyde-3-phosphate dehydrogenase (GAPD)
H60514 Lactate dehydrogenase A (LDHA)
AA599187 Phosphoglycerate kinase 1 (PGK1)
AA663983 Triosephosphate isomerase 1 (TPI1)

Metabolism—other

T39245 Methionine adenosyltransferase II alpha (MAT2A)
AA487666 Guanine deoxycarboxylase antisense 1 (OAZ1)
RS0991 Spermindine, spermine N1-acetyltransferase (SAT)
AA679907 Isochoreate dehydrogenase (IDH2)
AA487521 Mitochondrial glutamic-oxaloacetic transaminase 2 (GOT2)
R15814 Mitochondrial coenzyme A oxidase 1 (COX1)
AA676223 Acyl-Coenzyme A oxidase 3 pristanoyl (ACOX3)
AA657950 Farnesyl diphosphate synthase (FDPS)
AA43558 Mitochondrial coenzyme A hydratase (ECHS1)
AA594031 Cytochrome c oxidase subunit Vb (COX4B1)
AA664931 Cytochrome c oxidase subunit VIC (COX6C)
AA662863 Cytochrome c oxidase subunit VIII (COX8)
RS3511 Cytochrome c (CYS)
AA111999 NADH dehydrogenase 1 alpha 1 (NDUFA1)
AA680322 NADH dehydrogenase alpha complex 4 (NDUFC4)
AA127094 NADH dehydrogenase Fe-S protein 8 (NDUFS8)
AA232263 NADH dehydrogenase flavoprotein 2 (NDUFB2)
AA663658 Ubiquinol-cytochrome c reductase core II (UCRRC2)
R12802 Ubiquinol-cytochrome c reductase core II (UCRRC2)
AA404486 Adenine nucleotide translocase 2 (ANT2)
AA663434 Adenine nucleotide translocase 3 (ANT3)
AA163059 Adenine nucleotide translocase 3 (ANT3)
AA044059 Voltage-dependent anion channel 1 (VDAC1)
AA484620 Mitochondrial phosphore carrier (PHC)

Signal transduction

R37953 Adenylyl cyclase-associated protein (CAP)
AA636086 Adenosine A3 receptor (ADORA3)
AA857163 Amphiphilin (AREG)
AA679177 Butyrate-induced transcript 1 (BINT1)
AA127094 Butyrate-induced transcript 1 (BINT1)
AA4288 Calumenin 2 (CALM2)
AA746758 Calumenin 2 (CALM2)
AA415935 Riboflavin binding factor 2-interacting factor (AP15L1)
AA43581 Guanidine nucleotide binding e stimulating 1 (GNB2L1)
Table 2

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<tr>
<th>Gene Description</th>
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<td>GDNF family receptor alpha 2 (GFRα2)</td>
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<td>AA43749</td>
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**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. 3A, left side, and Fig. 3B, 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. 3A, right side, and Fig. 3B, top) contained 14 during treatment samples, 4 pretreatment biopsies, and also had lower expression in the during treatment branch 2 (Node 5, right side, and Fig. 3B, 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. 3A, right side, and Fig. 3B, 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. 3A). 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. 2A; Node 2, Fig. 3A). Of particular interest was a large node containing 338 genes that also had lower expression in the during treatment branch 2 (Node 5, Fig. 3A). Within this node 297 genes exhibited significantly different expression between branch 1 and branch 2 (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. 3A; 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. 3C, bottom, and Fig. 3D, 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. 2A). 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 prior to 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 methylentetrahydrofolate 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
ACKNOWLEDGMENTS

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REFERENCES


Molecular Pharmacology of Cancer Therapy in Human Colorectal Cancer by Gene Expression Profiling

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