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Comparison of gene expression in HCT116 treatment derivatives generated by two different 5-fluorouracil exposure protocols

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Abstract

Background: Established colorectal cancer cell lines subjected to different 5-fluorouracil (5-FU) treatment protocols are often used as *in vitro* model systems for investigations of downstream cellular responses to 5-FU and to generate 5-FU-resistant derivatives for the investigation of biological mechanisms involved in drug resistance. We subjected HCT116 colon cancer cells to two different 5-FU treatment protocols in an attempt to generate resistant derivatives: one that simulated the clinical bolus regimens using clinically-achievable 5-FU levels, the other that utilized serial passage in the presence of increasing 5-FU concentrations (continuous exposure). HCT116 Bolus3, ContinB, and ContinD, corresponding to independently-derived cell lines generated either by bolus exposure or continuous exposure, respectively, were characterized for growth- and apoptosis-associated phenotypes, and gene expression using 8.5 K oligonucleotide microarrays. Comparative gene expression analyses were done in order to determine if transcriptional profiles for the respective treatment derivatives were similar or substantially different, and to identify the signaling and regulatory pathways involved in mediating the downstream response to 5-FU exposure and possibly involved in development of resistance.

Results: HCT116 ContinB and ContinD cells were respectively 27-fold and >100-fold more resistant to 5-FU and had reduced apoptotic fractions in response to transient 5-FU challenge compared to the parental cell line, whereas HCT116 Bolus3 cells were not resistant to 5-FU after 3 cycles of bolus 5-FU treatment and had the same apoptotic response to transient 5-FU challenge as the parental cell line. However, gene expression levels and expression level changes for all detected genes in Bolus3 cells were similar to those seen in both the ContinB (strongest correlation) and ContinD derivatives, as demonstrated by correlation and cluster analyses. Regulatory pathways having to do with 5-FU metabolism, apoptosis, and DNA repair were among those that were affected by 5-FU treatment.

Conclusion: All HCT116 derivative cell lines demonstrated similar transcriptional profiles, despite the facts that they were generated by two different 5-FU exposure protocols and that the bolus exposure derivative had not become resistant to 5-FU. Selection pressures on HCT116 cells as a result of 5-FU challenge are thus similar for both treatment protocols.

Background

5-FU is one of the standard drugs used in chemotherapeutic regimens for metastatic colorectal cancer worldwide, despite the fact that resistance to 5-FU is a major obstacle to successful therapy. Elucidation of the complex biological mechanisms involved in development of resistance to 5-FU is an essential step towards predicting or overcoming such resistance. Established colorectal cancer cell lines subjected to different 5-FU treatment protocols are often used as *in vitro* model systems for investigations of downstream cellular responses to 5-FU and to generate 5-FU-resistant derivatives for the study of biological mechanisms involved in selection for drug resistance. We used the HCT116 colon cancer cell line for investigations of response to 5-FU and development of resistance because this cell line induces high and dose-dependent levels of apoptosis in response to increasing concentrations of 5-FU and can thus be considered to be sensitive to 5-FU. We generated treatment-derivative cell lines from parental HCT116 cells subjected to two different 5-FU treatment protocols. One protocol simulated the clinical bolus regimens using clinically-achievable 5-FU levels, the other utilized serial passage of HCT116 cells and continuous exposure to increasing 5-FU concentrations. The latter method is the more traditional one used to generate drug resistance, but one drawback is that such protocols often use higher, potentially clinically-irrelevant drug concentrations. The in vitro bolus 5-FU exposure protocol used was based on a previously-published protocol designed to simulate clinical bolus regimens in order to generate treatment-derivative cell lines [1]. This was achieved by a step-wise dilution of medium after 5-FU addition that was timed to approximate clinical clearance kinetics. It was assumed that this protocol would generate 5-FU-resistant derivatives of HCT116 after only three cycles of treatment, since it was previously shown to do so for two other colorectal cell lines, HT29 and HCT8. We thus opted to use three cycles of bolus treatment and to subsequently characterize the resultant derivative(s) generated regardless of resistance status at treatment endpoint.

5-FU resistance phenotypes were assessed in the treatment derivatives generated by both exposure protocols using growth inhibition and apoptosis assays. We also compared gene expression in the HCT116 treatment-derivatives generated by both 5-FU exposure protocols in order to determine whether the signaling and regulatory pathways involved in mediating the downstream response to 5-FU exposure (and possibly involved in development of resistance) were similar or substantially different as a result of differential treatment. Gene expression in the treatment derivatives and parental cells was detected and quantified using oligonucleotide microarrays. Microarray technology facilitates a more complete and inclusive experimental approach whereby alterations at the transcript level can be simultaneously measured for entire genomes in response to a defined stimulus, e.g. drug treatment [2]. This allows for a more rapid determination of whether specific signaling and/or regulatory pathways have in fact been affected in common for all cell lines examined, or if they are substantially different from one cell line to another. The results of our investigations demonstrate that there were strong similarities in gene expression among all the resultant treatment-derivatives despite the fact that they were generated by two different treatment protocols, and despite the fact that the bolus derivative, unlike the continuous exposure derivatives, had not become resistant to 5-FU after three cycles of treatment. Selection pressures on HCT116 cells as a result of 5-FU challenge are thus similar for both treatment protocols.

Results

Assessments of growth- and apoptosis-associated phenotypes in HCT116 parental and derivative cells

The drug concentration that resulted in a 50% growth inhibition (GI_{50}) was determined graphically from sigmoidal dose-response curves of log-transformed dose values versus cell counts (as percent of control) following 24 hours of continuous drug exposure. These were used to calculate resistance levels in the treatment-derivatives. The GI_{50} values for 5-FU were previously determined to be 0.09 mM for the parental HCT116 line, 2.4 mM for the ContinB line, and 10.9 mM for the ContinD line, indicating that the ContinB and ContinD derivatives were respectively 27-fold and >100-fold more resistant to 5-FU than the sensitive parental HCT116 cell line [3]. Following 3 cycles of bolus treatment, the HCT116 Bolus3 cell line was evaluated for its 5-FU resistance level compared to the parental cells. The GI_{50} concentration for 5-FU for the HCT116 Bolus3 cell line was determined to be 0.0022 mM, indicating that this derivative had not become resistant to 5-FU after 3 cycles of bolus treatment (plots not shown). Despite the fact that it had not become resistant, we proceeded to further characterize Bolus3 cells in order to gain valuable information about the early cellular responses to 5-FU.

Transient challenge with several different concentrations of 5-FU resulted in significantly lower apoptotic fractions for the ContinB and ContinD derivatives, respectively, compared to the sensitive parental cells at 24 hours (Figure 1). The Bolus3 derivative responded to the same 5-FU concentrations by inducing dose-dependent levels of apoptosis; the apoptotic fractions at 24 hours were very similar to those seen in the parental cell line (Figure 1).

Gene expression in the HCT116 derivatives compared to the parental line

The raw gene expression data for all of the 8500 probe sets on the microarrays for each cell line hybridization have

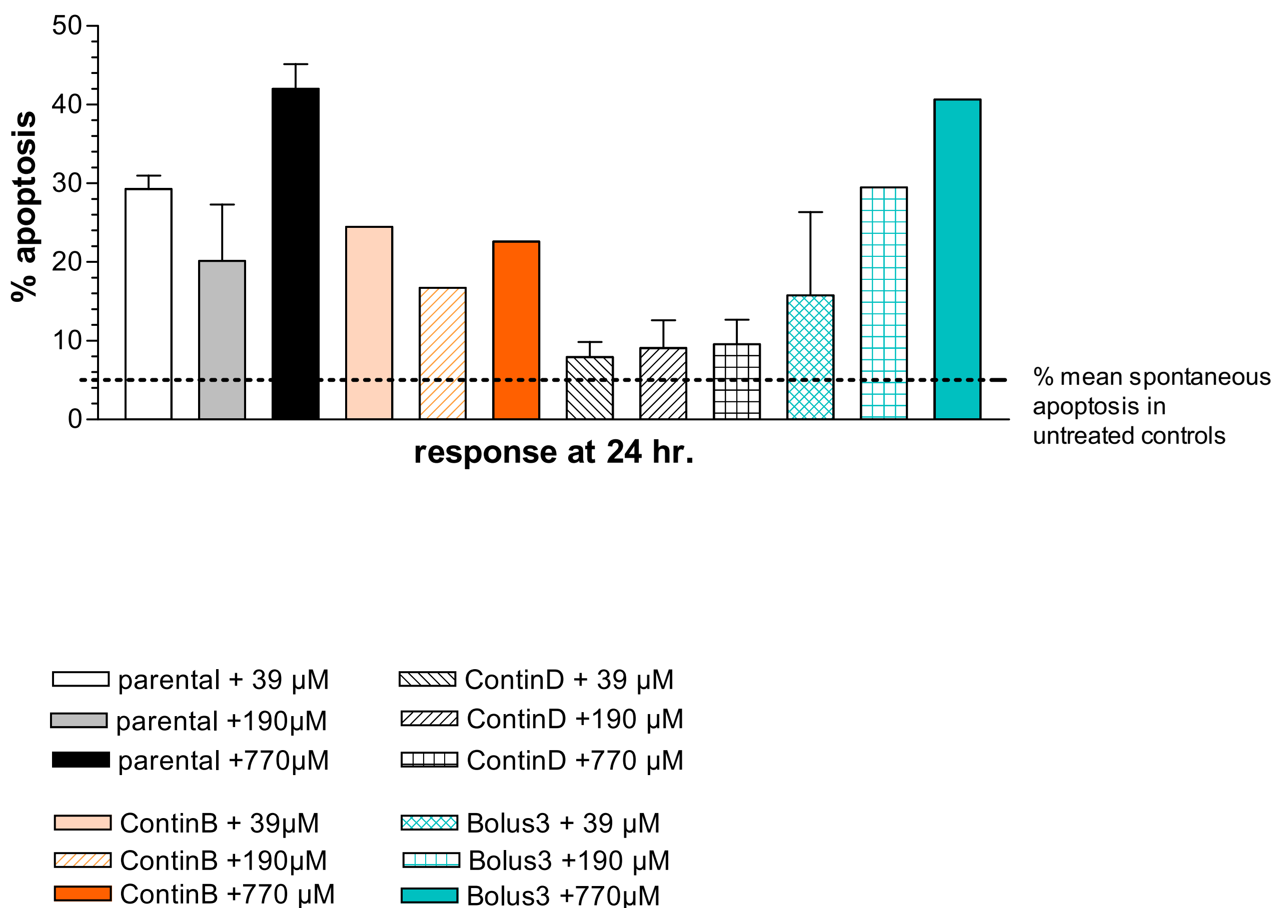

Figure 1
Apoptotic response of HCT116 parental and treatment-derivatives to transient 5-FU challenge for 24 hours. 5-FU was continuously present in the media of exponentially-growing cells for 24 hours. Apoptotic fractions were measured using the TUNEL assay as outlined in Materials and Methods. % apoptosis refers to the number of TdT-positive cells, i.e., cells that have been end-labeled with a biotin-labeled nucleotide and counterstained with a streptavidin-conjugated FITC fluorochrome.

not been presented in this study, but they are available upon written request. For individual parental HCT116, Bolus1, Bolus3, ContinB, and ContinD cell line hybridizations, 51%, 53%, 53%, 52%, and 53% of the total number of genes on the arrays were detected as present, respectively. Marginal expression was detected for 1–2% of all genes on the arrays for each cell line. Since the individual hybridized arrays were to be compared for gene expression levels, the scale factors for each hybridized array when compared to each other had to demonstrate less than a 3-fold difference, as recommended by Affymetrix. The hybridized arrays in the present study met these criteria. There was excellent agreement between 2 replicate array hybridizations for the ContinD cell line ($r = 0.980$, $p = 0.00$), and between Affymetrix oligonucleotide microarray and RT-PCR assessments of mRNA level changes for all 4 genes tested ($r = 0.940$, $p = 0.001$, data not shown).

Correlation analyses and hierarchical clustering of gene expression data

Correlation analyses of raw signal intensity values (gene expression levels) demonstrated strong correlations between each pair of cell lines tested (correlation coefficients typically greater than 0.90), but the strongest correlations were demonstrated between the Bolus3 and ContinB derivatives, the Bolus3 and Bolus1 derivatives,

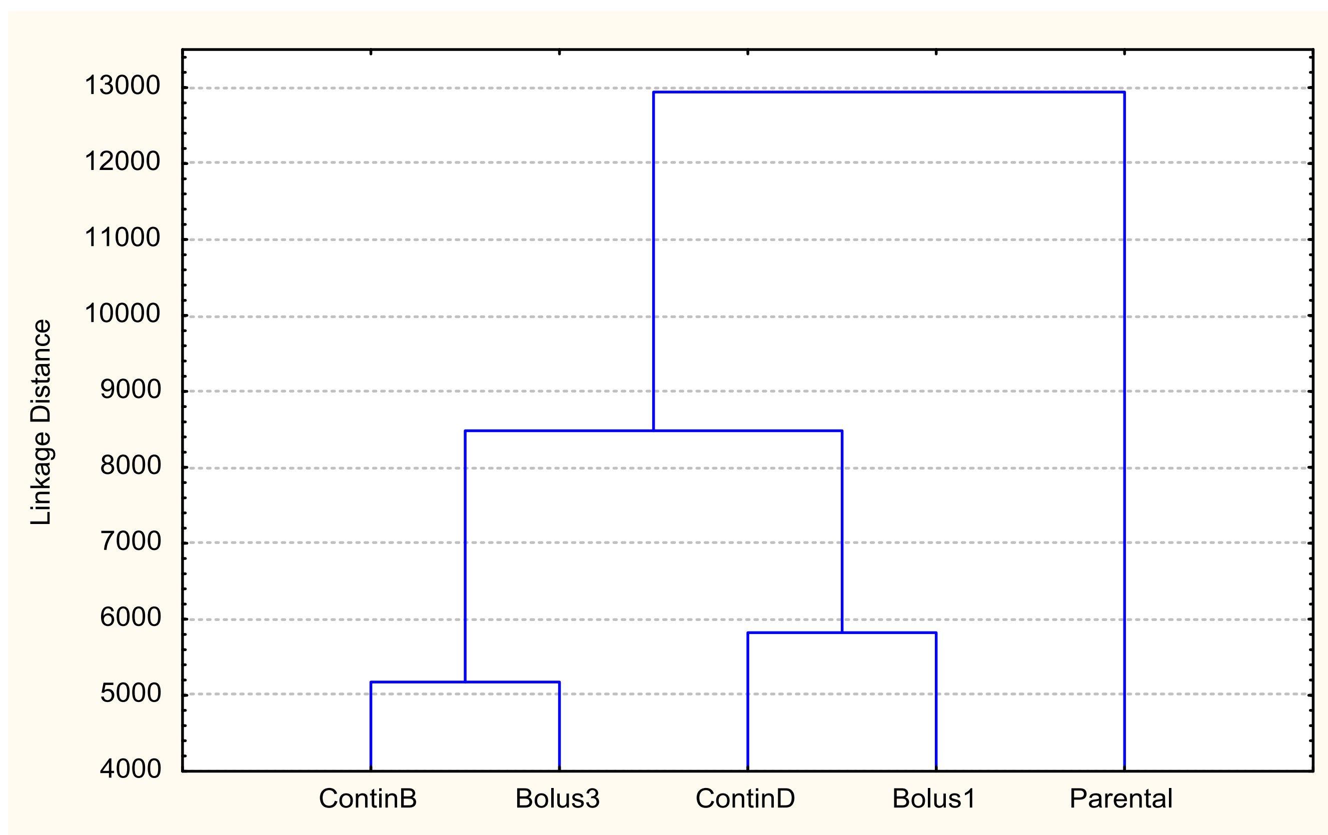

Figure 2
Cluster diagram showing correlations among all HCT116 cell lines for probe signal intensities (gene expression levels) for all genes examined. The pairs of cell lines that are connected tend to have the same gene expression level patterns, e.g., elevated gene expression level for one gene in one cell line tends to imply elevated gene expression level in the other cell line for the same gene. The length (linkage distance) of the vertical bars on the cluster plot indicates the degree of difference in gene expression level patterns between cell lines, e.g., the ContinB/Bolus3 and ContinD/Bolus1 cell line pairs were more similar to each other than either of these pairs was to the parental line for this parameter.

and the ContinD and parental cell lines, indicating that these pairs of cell lines tended to have the same gene expression level patterns (Table 1). For example, if one cell line in a correlated pair of cell lines had an elevated expression level for a particular gene, the other cell line tended to have an elevated expression level for the same gene, and vice versa, if one cell line had a low gene expression level for a particular gene, the other cell line tended to have the same. However, the actual signal intensity values were not exactly the same for each gene in a correlated pair of cell lines, although in some cases they could be. Cluster analysis performed for all cell lines using signal intensity values demonstrated groupings between ContinB and Bolus3, and between ContinD and Bolus1 (Figure 2). The length of the vertical bars on the cluster plot indicates the degree of difference in gene expression level patterns between cell lines, showing that the ContinB/Bolus3 and ContinD/Bolus1 cell line pairs were more similar to each other than either of these pairs was to the parental line for this parameter. ContinB and Bolus3 derivatives and Bolus3 and Bolus1 derivatives were strongly correlated for the signal $\log_2$ ratio parameter, demonstrating that gene expression changes in the derivative cells (relative to the parental cells against which they were compared) were very similar in these pairs of cell lines. The ContinD derivative demonstrated moderate correlations with the Bolus3 derivative, with the ContinB derivative, and with the Bolus1 derivative for the same parameter (Table 1), indicating that gene expression changes were more dissimilar in these pairs of cell lines. The length of the vertical bars on the cluster plot in Figure 3 (cluster analysis for signal $\log_2$ ratios) indicates the degree of difference in gene expression changes between cell lines, showing that the ContinB/Bolus3 and ContinD/

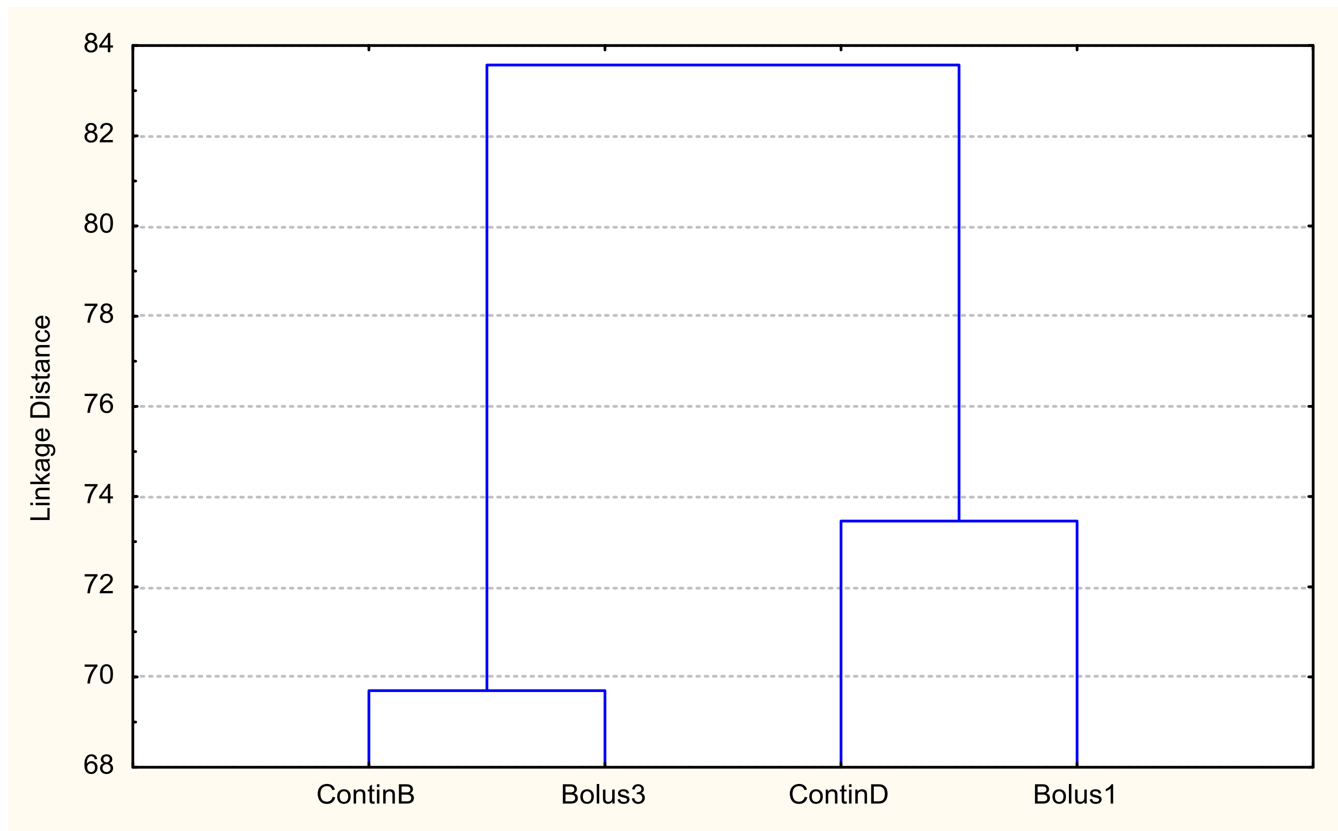

Figure 3
Cluster analysis of signal $\log_2$ ratios (gene expression changes) for four derivative cell lines. Similar gene expression changes are demonstrated in the grouped pairs of treatment-derivatives, varying in degree of difference according the linkage distance. The smaller the linkage distance, the more similar the gene expression changes. ContinB and Bolus3 derivatives had similar gene expression changes, as did ContinD and Bolus1 derivatives.

Table 1: Correlation analyses for signal intensity values (gene expression levels) and signal $\log_2$ ratios (gene expression changes) in HCT116 derivative and parental cells, using microarray expression data for all genes.

Pair of variables		Gene expression levels (r-values)	Gene expression changes (r-values)
Bolus3	and Bolus1	0.970	0.603
	and ContinB	0.971	0.674
	and ContinD	0.957	0.457
	and parental	0.947	
Bolus1	and ContinB	0.963	0.531
	and ContinD	0.963	0.382
	and parental	0.959	
ContinB	and ContinD	0.954	0.428
	and parental	0.944	
ContinD	and parental	0.968	

Results of correlation analyses are presented as correlation coefficients (r-values). All correlations were significant, with p-values = 0.00.

Table 2: Examples of some of the regulatory pathways affected by 5-FU exposure, and associated genes that were scored as up- or down-regulated in one or more HCT116 treatment-derivatives.

Regulatory pathway affected	Altered gene in one or more derivatives	Bolus3 signal log_2 ratio	ContinB signal log_2 ratio	ContinD signal log_2 ratio
5-FU-metabolism	*DHFR*	0.6	0.7	1.0
	DPYD	-1.0	-1.5	-0.9
	DTYMK	1.0	1.4	0.7
	TYMS	0.7	0.9	0.7
	UP	1.2	1.6	(0.3)
Apoptosis	*ANXA4*	-1.0	-1.2	(-0.3)
	BIRC5	0.6	1.2	0.7
	BNIP3L	-2.4	-2.7	-0.5
	IRAK1	1.4	1.6	0.6
	MALT1	0.6	1.5	0.8
	PDCD4	-2.8	-2.9	(0.0)
DNA repair	*FANCG*	2.1	2.3	1.7
	FEN1	1.5	1.7	1.1
	RAD23B	2.3	2.3	(0.2)

Signal log_2 ratios $\geq$ 0.5 defined an up-regulated gene, and signal log_2 ratios $\leq$ -0.5 defined a down-regulated gene. Values in parentheses represent 'no change' scores and are included here for comparison.

Bolus1 cell line pairs were again more similar to each other than either of these pairs was to the other pair for this parameter.

Specific Affymetrix software filters were applied to the gene expression data to provide information about the regulatory pathways affected as a result of treatment (e.g. apoptosis and DNA repair); Table 2 shows some of the affected regulatory pathways and the corresponding genes scored as up- or down-regulated in one or all of the HCT116 treatment-derivatives.

Discussion

We compared gene expression and specific growth- and apoptosis-associated phenotypes in HCT116 treatment-derivatives generated either by continuous exposure to 5-FU or by using an exposure protocol that more directly simulated the clinical bolus regimens. The aim was to determine whether the signaling and regulatory pathways involved in mediating the downstream response to 5-FU exposure were similar or substantially different as a result of these two different treatment protocols. A previous study [1] attempted to systematically address this question by determining the activity of different enzymes involved in 5-FU metabolism in cell lines generated by these two methods, and demonstrated differences in enzyme activity levels between the respective resultant derivatives. However, the authors did not have DNA microarray technology available to them at that time in order to gain complete transcriptional profiles of the cell lines used in their study. Use of this technology in the present study yielded comparative information about expression levels simultaneously for the same 8500 genes for three derivative cell lines and one parental cell line (baseline) from which the other three were derived, allowing for a more rapid determination of whether specific signaling and/or regulatory pathways had in fact been affected in common for all derivative cell lines examined, or if they were substantially different from one cell line to another.

All derivative cell lines demonstrated similar transcriptional profiles, despite the facts that they were generated by two different 5-FU exposure protocols and that they differed substantially in their levels of resistance to 5-FU. This suggests that similar signaling and regulatory pathways were involved in mediating the downstream response to 5-FU in each of the derivative lines, i.e., that the selection pressures on the cells in terms of dealing with 5-FU challenge are similar for both exposure protocols. Despite the fact that use of the in vitro bolus model for resistance development did not result in 5-FU-resistant derivatives after 3 cycles of treatment, we opted to proceed with characterizations of the resultant bolus derivatives generated. We reasoned that these derivatives could provide valuable information about the early downstream responses to 5-FU. The Bolus3 derivative had a transcriptional profile that was very similar to that seen in the strongly-resistant ContinB derivative. The actual genes selected for and expressed in response to 5-FU exposure, and the levels of gene expression were very similar between the two cell lines; additionally, many of the same genes were scored as up- or down-regulated in both derivatives. The Bolus3 and Bolus1 derivatives were also

strongly correlated for gene expression levels and gene expression level changes, not surprisingly since the Bolus3 derivative is a simply a sequential continuation of the Bolus1 cell line, differing only in the number of bolus cycles it has undergone. Gene expression analysis of Bolus1 cells was included in the study in order to gain valuable information about the transcriptional profile associated with one cycle of 5-FU treatment and recovery. The results indicate that already after one round of 5-FU treatment and recovery that these cells had a transcriptional profile that was very similar to that seen in the Bolus3 and ContinB cells. The ContinD derivative demonstrated strong correlations with the ContinB, Bolus3 and Bolus1 derivatives for gene expression levels, but only moderate correlations with the ContinB, Bolus3 and Bolus1 derivatives for gene expression changes. A strong correlation was demonstrated with a replicate ContinD sample for gene expression levels, indicating a high degree of reproducibility of the data. All HCT116 treatment-derivative lines also demonstrated gene expression that was very similar to the parental HCT116 cell line, reflecting their origin from this cell line. However, the Bolus 3 and ContinB cell lines were the treatment-derivatives that differed most from the parental line, yet were most like each other. The ContinD derivative demonstrated the strongest correlation with the parental HCT116 cell line with regard to gene expression levels of all the derivatives, followed by the Bolus1 derivative, thus the ContinD derivative is most like the parental line. This was also demonstrated by cluster analysis using gene expression level data for all cell lines. The ContinD line clustered together with the Bolus1 line, whereas the ContinB and Bolus3 lines clustered together; the parental HCT116 cell line clustered together with each of these individual clusters, but was much closer distance-wise to the ContinD/Bolus1 cluster. Further analysis of the gene expression data for all of the HCT116 treatment-derivatives, with specific focus on the differences in gene expression between these cell lines, will lead to an explanation of what defines resistance to 5-FU in these cells. We conclude that the ContinB and ContinD derivatives can be useful *in vitro* models for the study of the molecular mechanisms underlying 5-FU resistance, whereas the Bolus3 derivative, while not resistant to 5-FU, can be useful for the study of early cellular responses to 5-FU exposure.

Recent articles about resistance to anticancer agents have discussed the importance of drug target alterations, DNA repair, and evasion of apoptosis as relevant mechanisms of drug resistance [4,5]. In the present study, genes on signaling and regulatory pathways associated with 5-FU metabolism, apoptosis, and DNA repair were among the many genes shown to be significantly altered during selection for 5-FU resistance. The main mechanism of action of 5-FU is to inhibit thymidylate synthase, a key enzyme in the de novo synthesis of dTMP from dUMP [2,6]. Cells continually exposed to 5-FU must compensate for the inhibition of thymidylate synthase and deal with the DNA damage / eventual apoptosis that may result due to FdUTP misincorporation into DNA. Overexpression of the thymidylate synthase (*TYMS*) gene is a characteristic of 5-FU-resistant cell lines [7,8] and is predictive of poor response to 5-FU treatment in colorectal cancer [9,10]. The *TYMS* gene was overexpressed nearly 2-fold in all of the HCT116 treatment-derivatives in the present study compared to the parental cells. This suggests that up-regulation of *TYMS* is one of the first cellular responses to repeated 5-FU exposure, but that it may not necessarily define a 'resistance' phenotype, since the Bolus3 derivative, while not 'resistant' to 5-FU as assessed by growth inhibition assays, expressed levels of this gene similar to those seen in the strongly-resistant ContinB and ContinD derivatives. Several other genes involved in 5-FU metabolism (e.g. *DTYMK, UP, DHFR*) were also up-regulated in both the Bolus3 derivative and in one or both of the continuous derivatives, suggesting that their overexpression contributes to but may not necessarily define a 'resistance' phenotype.

Since we have a documented reduced apoptosis phenotype for the ContinB and ContinD derivatives (but not for the Bolus3 derivative), we conclude that loss of sensitivity to apoptosis induction accompanies selection for resistance to 5-FU in these cell lines. This phenotype cannot be explained by loss of wild-type p53 function, since both the ContinB and ContinD cell lines retained a wild-type *TP53* genotype [3], and both cell lines induce p53 in response to transient 5-FU challenge in similar fashion to the parental cell line. It was thus relevant to examine expression levels of other genes involved in the regulation of apoptosis. Genes involved in apoptosis promotion, e.g., *ANXA4*, *BNIP3L*, and *PDCD4*, were strongly down-regulated in the ContinB and Bolus3 derivatives (but not in the ContinD derivative) in the present study. Genes involved in apoptosis inhibition, e.g. *BIRC5* and *MALT1*, were strongly up-regulated in the ContinB derivative but not in the Bolus3 or ContinD derivatives, whereas *IRAK1* was strongly up-regulated in both the ContinB and Bolus3 derivatives, but not in the ContinD derivative. Overexpression of *BIRC5*, *MALT1*, and *IRAK1* may thus contribute to the reduced apoptosis phenotype in the ContinB derivative cell line, but other (still unknown) apoptotic-regulatory genes might account for the same in the ContinD derivative. Overall, our results are consistent with recent publications that suggest that overexpression of apoptosis-inhibitory genes or underexpression of apoptosis-promoting genes contribute to a drug resistance phenotype [4,5], at least for the ContinB derivative.

Likewise, DNA repair genes such as *FEN1* and *FANCG* were overexpressed in both continuous-treatment

derivatives, suggesting that the latter can repair DNA more effectively than the parental line, consistent with recent studies that suggest that DNA repair contributes to general drug resistance [4,5]. It is unknown at present whether overexpression of these genes results in more efficient DNA repair since we have not measured DNA repair activity. The ContinB derivative also overexpressed *RAD23B*. The Bolus3 cell line also overexpressed all three of these DNA repair genes at levels similar to those measured for the continuous derivatives, despite its lack of resistance to 5-FU. An investigation of what underlies the strong 5-FU resistance demonstrated in the ContinB and ContinD cells must therefore focus on other highly-up-regulated or down-regulated genes and their levels of expression, as well as on the alterations that have occurred at the gene level (e.g. increase in copy number). These will involve both functional studies using gene silencing and the use of genomic arrays, respectively, and this work is currently underway in our laboratory.

The bolus exposure regimen employed in the present study was previously shown to generate resistant cell populations after only 3 cycles of treatment in two different colorectal cancer cell lines, HCT8 and HT29 [1], but this was not the result for the HCT116 cells used in our study. One probable explanation for this is that HCT116 cells may be more difficult to make resistant to 5-FU than HCT8 and HT29 cells, and that it would take exposure to higher concentrations of the drug or to increased numbers of treatment cycles in order to achieve resistance. The fact that HCT116 cells are DNA-mismatch repair-deficient due to lack of hMHL1 expression may explain their tolerance to 5-FU, since loss of DNA mismatch repair has been shown to confer resistance to 5-FU [11,12]. It has been shown that lack of hMHL1 expression is the result of hypermethylation of the hMHL1 gene, and a recent publication demonstrated that it was possible to restore sensitivity to 5-FU (in effect, overcome *in vitro* resistance) by re-expression of hMHL1 protein through demethylation in hypermethylated cell lines [13]. We have not assessed the methylation status of this gene in any of the cell lines used in this study.

Conclusions

The present study demonstrates that downstream signaling and regulatory pathways affected by continuous 5-FU exposure were similar to those affected by clinically-relevant 5-FU exposure, i.e., that HCT116 treatment-derivatives generated by two different 5-FU exposure protocols have similar transcriptional profiles. The selection pressures on HCT116 cells as a result of 5-FU challenge can thus be considered to be similar for both types of treatment protocols. HCT116 treatment-derivatives generated by continuous exposure to 5-FU can be useful *in vitro* models for the study of the molecular mechanisms underlying 5-FU resistance because they provide information about the types of signaling and regulatory pathways affected by repeated exposure to 5-FU. The HCT116 Bolus3 derivative, while not resistant to 5-FU, can be useful for the study of early cellular responses to 5-FU exposure. The specific differences between these derivatives in terms of the types of genes expressed and the expression levels of these genes will aid in the elucidation of factors that define resistance to 5-FU. Finally, the stability of the resistance phenotypes in the strongly-5-FU-resistant ContinB and ContinD cell lines are most likely due to genotypic alterations, and future studies will focus on determining the types of alterations that have occurred at the gene level through the use of genomic arrays, and the point during resistance development at which they occurred.

Methods

Cell lines, culture conditions and chemicals

Early passage HCT116 cells (ATCC CCL 247) derived from a poorly-differentiated colonic adenocarcinoma were used. These cells have a wild-type *TP53* genotype, a somatic frameshift mutation in exon 3 of the *BAX* gene [14,15], DNA-mismatch repair-deficiency due to lack of hMHL1 expression [16], two different thymidylate synthase enzymes [17,18] and a near-diploid DNA complement. Cells were maintained as monolayers in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 0.6% Pen-Strep at 37°C in a 5% CO_2 atmosphere (all cell culture reagents and plasticware purchased from Invitrogen Gibco). 5-FU was obtained from Amersham Life Sciences, England.

Serial passage of HCT116 cells in increasing 5-FU concentrations (continuous exposure)

Derivative cell lines were generated by serial passage of parental HCT116 cells in the presence of increasing concentrations of 5-FU, and recently characterized for various phenotypes and genotypes [3]. Briefly, intermittent 5-FU treatments of exponentially-growing HCT116 cells for 48 hours, followed by recovery periods in drug-free medium until the cells regained exponential growth (an interval corresponding to circa 18 doubling times), were used to generate independent treatment derivatives. The concentrations of 5-FU used ranged from 5–770 μM. Two independent derivatives, designated ContinB and ContinD (original designation ResB and ResD; designation changed for the present study in order to emphasize difference between derivatives generated by continuous 5-FU exposure versus those generated by bolus treatment), were selected and cultured further. Drug treatments continued until both derivative cell lines demonstrated stable resistance to 5-FU challenge, as assessed by growth inhibition assays.

Bolus 5-FU exposure of HCT116 cells (clinical clearance kinetics model)

Pizzorno and Handschumacher [1] used 5-FU concentrations that were within the achievable clinical range to treat two different colon cancer cell lines, using an exposure protocol that more directly simulated the clinical regimens currently in use. The bolus protocol provided a drug concentration profile similar to that obtained in patients after a 600 mg/m^2 dose of 5-FU [1]. This was achieved by a stepwise dilution of medium after 5-FU addition that was timed to approximate clinical clearance kinetics. The initial 5-FU concentration of 500 μM was diluted out to 250 μM, 100 μM, 20 μM, 2 μM and finally to 0.5 μM over the course of approximately 5 hours. Cells were then allowed to incubate in 0.5 μM 5-FU for 24 hours. The following day, the same regimen was repeated. This protocol was repeated daily for 5 consecutive days, at which point the cells were removed to drug-free media via a medium shift and allowed to recover. The entire regimen was repeated 3 times at intervals corresponding to about 18 doubling times [1]. We performed 3 cycles of treatment since these authors demonstrated that their exposure regimen resulted in the selection of (transiently)-resistant cell populations after only three cycles of treatment in two other colorectal cancer cell lines, HCT8 and HT29. Cell cultures that had recovered following each treatment cycle were frozen down for subsequent gene expression and growth inhibition analyses; these were labeled as HCT116 Bolus1, Bolus2, and Bolus3. Assessments of growth inhibition were done only for HCT116 Bolus3, since this derivative should in principle have developed the most resistance to 5-FU after three cycles of treatment. Gene expression levels were quantified for both HCT116 Bolus3 and Bolus1 cells; gene expression in the latter was used as a basis for comparison with the same in HCT116 Bolus3 cells. Untreated exponentially-growing parental HCT116 cells were used as controls.

Subsequent characterizations of all HCT116 derivative cell lines were done on exponentially-growing cells that had been grown without 5-FU in the media for at least one month, taking into account recommendations for *in vitro* resistance work presented in a recent review [2].

Assessment of resistance levels in HCT116 parental and treatment-derivative cell lines using growth inhibition assays

HCT116 parental and treatment-derivative cells that were maintained in regular medium without 5-FU were seeded in duplicate in 12-well plates and allowed to grow until they reached early exponential growth. Media was shifted 24 hours before the addition of 5-FU. Exponentially growing cells were incubated in the absence or presence of 8–10 different 5-FU concentrations for 24 hours, at which time the medium was aspirated off, the cells trypsinized and counted using a trypan blue viability assay. The drug concentration that resulted in a 50% growth inhibition (GI_{50}) was determined graphically from sigmoidal dose-response curves of log-transformed dose values versus cell counts (percent of control) at 24 hours of continuous drug exposure. The resistance level was calculated as the ratio between the GI_{50} concentrations of the drug in the growth medium of the resistant and the sensitive cells, respectively. Two replicate experiments were performed.

Quantification of apoptotic response to 5-FU in HCT116 parental and treatment-derivative cell lines

Apoptotic response to transient 5-FU challenge was quantified in the parental and treatment-derivative HCT116 cell lines at 24 hours following 5-FU addition to the medium, using the TUNEL method for apoptosis detection. Different concentrations of 5-FU were added to the medium of exponentially-growing cells that had otherwise been maintained in 5-FU-free medium for one month. Apoptotic cells in control and treated samples were identified by end-labeling apoptotic DNA fragments with a fluorescent marker (streptavidin-FITC labeling of incorporated biotin-conjugated dUTP), mediated by terminal deoxynucleotidyl transferase (TdT) [19]. Cells were counterstained with 5 μg/ml propidium iodide to stain cellular DNA. The resulting labeled apoptotic fractions were quantified in the control and treated cell samples using a FACSCalibur laser flow cytometer (BDIS, San Jose, CA), after appropriate gating using pulse-width analysis of the DNA content signal to exclude doublets and aggregates. Two replicate experiments were performed.

Oligonucleotide array analyses and quantification of gene expression levels

Harvesting of the cells for RNA extraction for comparative investigations of gene expression in the treatment-derivative and parental HCT116 cell lines was done on exponentially growing cells that were maintained in regular medium without 5-FU at the time of the actual microarray investigations. Analyses were done using Gene Chip Human Genome 8.5 K Focus Arrays (Affymetrix, Santa Clara, CA). Information about these arrays can be found at http://www.affymetrix.com. RNA isolation, target labeling and hybridization to the arrays, array washing, staining, scanning and data analysis were performed using Affymetrix standard protocols, instrumentation, and Microarray Suite version 5.0 software. One replicate hybridization was performed for the ContinD derivative (same RNA sample, labeled anew and hybridized) in order to confirm the reproducibility of gene expression alterations between two same sample hybridizations.

The Microarray Suite software contains Detection, Signal, Change, and Signal log ratio algorithms that are used to evaluate individual gene expression for all gene probe sets

(including controls) on the microarrays. These algorithms are described in detail in the *Statistical Algorithms Description Document* published by Affymetrix and available on their website. Briefly, the Detection algorithm assesses probe pair saturation, calculates a detection p-value, and assigns a present (P), marginal (M), or absent (A) call. The Signal algorithm calculates a quantitative metric for each probe set using the stray and real signal hybridization intensities, corresponding to Mismatch (MM) and Perfect Match (PM) minus MM, respectively. For comparison analysis of two samples (in the present study, a treatment derivative versus the parental cell line), a Change algorithm is used to generate a change p-value and an associated 'change', which indicates whether gene expression is increased (I), marginally-increased (MI), decreased (D), marginally-decreased (MD) or no change (NC) in a resistant derivative compared to the parental cell line. The Signal log ratio algorithm produces a quantitative estimate of these gene expression changes in the form of signal $\log_2$ ratios.

Correlation and cluster analyses for HCT116 derivative and parental cell lines were done for the following parameters: a) raw signal intensity values: these values are actual gene expression levels, and were compared for all genes in all cell lines (derivatives and parental) using Spearman correlation analysis; b) signal $\log_2$ ratios: these values are quantitative estimates of gene expression changes, and were compared using Spearman correlation analysis. Hierarchical cluster analyses for the same parameters were done using the Joining:Tree clustering method, Complete linkage, distance metric was Euclidean distances.

Real-time RT-PCR

Alterations of gene expression detected using Gene Chip arrays were confirmed by real-time RT-PCR for 4 genes, *TYMS, F3, FEN1,* and *TOP2A*, in ContinB and ContinD cells. These genes were chosen for RT-PCR analysis because they were shown to be strongly up- or down-regulated by microarray analysis in ContinB and/or ContinD cells [3]. The *GAPDH* gene was used as an internal control. Information about the cDNA-specific primers and procedure used for these investigations are available in the same study.

Statistical analyses

Correlation and hierarchical cluster analyses were performed using Statistica version 5.5 software (StatSoft Inc., Tulsa, OK). p-values ≤ 0.05 were considered to denote statistical significance.

Authors' contributions

PMD conceived the study, carried out one-half of the resistance development work, evaluated the microarray data, and did the statistical analyses. KLK carried out all maintenance cell culture work and one-half of the resistance development work. Both PMD and KLK performed the growth inhibition assays and did the RNA isolations necessary for microarray work. SHT performed the oligonucleotide microarray hybridizations under supervision from TH, and WR performed real-time RT-PCR. All authors read and approved the final version.

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