5-Fluorouracil response in a large panel of colorectal cancer cell lines is associated with mismatch repair deficiency

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BACKGROUND: Colorectal cancer (CRC) is one of the commonest cancers and its therapy is still based on few drugs. Currently, no biological criteria are used to choose the most effective of the established drugs for treatment.

METHODS: A panel of 77 CRC cell lines was tested for sensitivity to 5-fluorouracil (5FU) using the SRB assay. The responses were grouped into three categories and correlated with genetic changes in the cell lines.

RESULTS: The strongest and most clearcut correlation was between 5-fluorouracil response and replication error status (mismatch repair deficiency). All the other significant correlations (loss of heterozygosity for DCC and mutations in TGFbRII) are secondary to the association with replication error status.

INTERPRETATION AND CONCLUSION: Our findings validate previous analyses based mainly on clinical data, and indicate that replication error status could be a useful guide to 5-fluorouracil-based CRC therapy. Essentially, all previously described correlations with 5FU response are secondary to the association with replication error status.

British Journal of Cancer (2010) 103, 340–346. doi:10.1038/sj.bjc.6605780 www.bjcancer.com
Published online 6 July 2010 © 2010 Cancer Research UK

Keywords: 5-fluorouracil; mismatch repair deficiency; replication error; colorectal cancer; cell lines

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in the western world (Rim et al, 2009), and the 5-year relative survival rate is still only between 44 and 60% in the United Kingdom and North America (Coleman et al, 2008). A wide range of genetic changes due to mutations and loss of heterozygosity (LOH), as well as epigenetic changes, are found in CRC, indicating the considerable heterogeneity of the disease. Involved genes include APC, KRAS, PIK3CA, BRAF, PTEN, SMAD4, MLH1, MSH2, TP53, and CTNNB1 (Sanger Institute). KRAS activating mutations have recently emerged as predictive biomarkers for treatment of CRC with EGFR inhibiting agents in clinical trials (Linardou et al, 2008; Riely and Ladanyi, 2008). Clinical data also suggest an association between 5-fluorouracil (5FU) response and RER status (Ribic et al, 2003; Warusavitarne and Schnitzler, 2007; Muller et al, 2008). These studies extended previous work on cell-based studies in isogenic pairs of HCT116 cells (Meyers et al, 2001; Arnold et al, 2003). So far, however, the KRAS association is the only predictive marker that is being used to guide the treatment of CRC.

Advances in the chemotherapy treatment of CRC are limited by the currently available selection of licensed drugs, most of which (oxaliplatin, 5FU, irinotecan, and capcitabine) have been in use for many years. The most recently introduced agents are antibodies targeting EGFR (cetuximab and panitumumab) or VEGF (bevacizumab). 5-Fluorouracil, which is also used in the treatment of breast, stomach, and pancreatic cancer, remains the cornerstone of CRC treatment, although widely used in combination with several other drugs (Kopetz, 2008). It is an antimetabolite that, after conversion into its main active metabolites (FdIMP, FdUTP, and FUTP), induces, among other effects, RNA and DNA damage through incorporation of its metabolites into nucleic acids, and inhibition of thymidylate synthase (TS) and therefore DNA synthesis (Longley et al, 2003). However, only 10–15% of advanced CRC tumours treated with 5FU/leucovorin first-line therapy respond (Longley et al, 2003; Chen et al, 2010), which highlights the need to establish predictive biomarkers for 5FU drug response.

We have accumulated in our laboratory a large collection of CRC-derived cell lines on which we have information regarding their genetic changes and variation in gene expression (see, e.g., Liu and Bodmer, 2006). This panel of cell lines provides a powerful tool for detecting associations between drug responses and the biological characteristics of the lines, which reflect the properties of the tumours from which they were derived. The use of the cell lines should, therefore, enable the discovery of new predictive markers for better patient selection and more targeted therapy.

In this study we present a robust method for large-scale screening of CRC cell lines directly from frozen stock and a simple objective approach to the classification of the cell lines with respect to their drug responses. Data from a screen of 5FU response in 77 cell lines were used for an analysis of correlations with genetic changes. The results show, in agreement with published clinical data, that the major genetic change affecting 5FU response is...
mismatch repair status, thus validating the appropriateness of our method of analysis.

MATERIALS AND METHODS

Cell lines
Details of the 77 cell lines used in this study are listed in Supplementary Table 1. Many of the lines have been described before (Liu and Bodmer, 2006). The cell lines were characterised with respect to mismatch repair deficiency (replication error status: RER + for mismatch repair defective and RER – otherwise (four microsatellite loci, BAT25, BAT26, D17S250, and D18S69, were amplified and used to determine RER status; Elston and et al., 1999)), mutations in APC (mutation cluster region), KRAS (codons 12 and 13), BRAF (V600E), TP53, CDH1 (E-cadherin), CTNNB1 (β-catenin; exons 3, 4, 5, and 6), MLH1, MSH2, PIK3CA (exons 9 and 20), B2M (β-2-microglobulin, exons 1 and 2), TGFB2 (TGFBIIIR), CHK1, and SMAD4, LOH around the genes APC and CDX1 (chromosome 5), SMAD4 and DCC (chromosome 18), and promoter methylation of MLH1, MSH2, DTCNA2 (p16), CDKNA2B (p15), and CDX1. Most cell lines have been HLA typed to identify duplicate cell lines, and all cell lines were routinely tested negative for mycoplasma.

Toxicity testing
Increased throughput was predominately achieved by plating cells directly into 96-well plates from frozen stock, thus negating the need for continuous culture of the cell lines. Optimal freezing and thawing conditions were established and the time required, after plating, for lines to begin their growth phase was determined and defined as the lag time. Standard freezing conditions involved suspending cells in 5% DMSO in FCS at a density of 4 × 10^6 cells per ml and placing them on dry ice for 90 min before transfer to liquid nitrogen. For use from frozen stocks, cells were warmed quickly to 37 °C and transferred immediately into prewarmed medium, centrifuged, and re-suspended in fresh, DMSO-free medium before use. Cell lines plated from thawed stocks were shown to have the same growth curves and drug dose-response curves as their cultured counterparts (data not shown). Lag times and doubling time (d.b.t.) for all cell lines after plating from frozen stock were established by plating 3000 cells per well and using the lag times shown to have the same growth curves and drug dose-response medium before use. Cell lines plated from thawed stocks were kept in liquid nitrogen. For use from frozen stocks, cells were warmed quickly to 37 °C and transferred immediately into prewarmed medium, centrifuged, and re-suspended in fresh, DMSO-free medium before use. Cell lines plated from thawed stocks were shown to have the same growth curves and drug dose-response curves as their cultured counterparts (data not shown). Lag times and doubling time (d.b.t.) for all cell lines after plating from frozen stock were established by plating 3000 cells per well and using the SRB assay as described below. The results are reported in Supplementary Table 1.

For toxicity testing, 3000 cells were plated in 100 μl medium in 96-well plates and incubated for their lag time before drug treatment with a further 100 μl of drug-containing medium in duplicate wells for each condition. Final concentrations of 5FU ranged from 0.13 to 100 μM (1:3 titration) and the final concentration of DMSO in all wells was 0.067%. Cells were incubated with drug for 3 d.b.t. to accommodate for differences in their growth rates, before they were fixed and stained following a standard SRB protocol (Vichai and Kirtikara, 2006). In brief, after spinning plates for 10 min at 1500 r.p.m., 50 μl of ice-cold trichloroacetic acid (2.6 M) was added and plates were incubated at 4 °C for 60 min. After washing the plates with 0.5 × PBSA, cells were stained with sulforhodamine B (0.04% in 1% acetic acid) for 1 h, and bound dye was dissolved in 200 μl Tris (10 mM, pH 9.5) before measuring the OD at 540 nm in a plate reader. T/T0% (‘treated over control (corrected)’ with a correction for the OD of the number of cells at the time of treatment) is calculated as (ODdrug/3.d.b.t. − OD0)/ODblank + OD0) × 100, and GI50 values were calculated from the resulting dose-response curves using model 210 (dose-response one site, five-parameter logistic model, fit = A + ((B − A)/(1 + x/(C−D)))^E)), and A and B unlocked in XLFit (IDBS, Surrey, UK). The experiment was repeated two to four times for each cell line.

Matrigel assay
A total of 700 cells were plated in 80 μl Matrigel (basement membrane matrix, growth factor reduced, BD (Oxford, UK), no. 356231) in 96-well plates and treated with 0, 0.5, 5, and 50 μM 5FU in duplicate wells each (150 μl per well added on top of Matrigel/cells). The medium was exchanged for fresh drug-containing medium on days 3, 6, 9, and 12 before colonies were counted under a light microscope (×10 objective) on day 14. Three fields of vision (FOV) were counted per well, averaging counts per FOV over both wells, and the experiment was repeated three times. Four cell lines were chosen for their known different abilities to differentiate in this matrix: LS174T and HT29 (intermediate differentiation), and LS180 and SW1222 (well differentiated).

Statistical analysis
The 77 cell lines were divided into terciles with respect to their sensitivity to 5FU: sensitive (n = 26), intermediate response (n = 26), and resistant (n = 25). To correlate drug response with categorical data (e.g., mutation status), we used χ2 association and trend tests in 2 × 2 and 3 × 2 tables for comparing categories of drug response with two genetic categories (e.g., wild type and mutant). For known duplicate cell lines, only one of each duplicate was counted for association studies. When drug response groupings differed between duplicate lines, both duplicates were omitted from the statistical analysis. Reported P-values were not corrected for multiple testing.

RESULTS

5FU drug response
Overall, GI50 values ranged from 0.03 (HDC73) to 47.5 μM (HCT15) in the 77 cell lines, giving an approximately 1600-fold maximum μM difference. Supplementary Table 2 lists the GI50 values for all the cell lines, including duplicates, in alphabetical order and the same data are shown in rank order in Figure 1. Examples of dose-response curves for three cell lines with different levels of 5FU sensitivity are given in Figure 2. The cell lines were divided into three sensitivity groups, ranging from sensitive (HDC73 to VAC010MS, 0.03–1.70 μM) to intermediate (HDC54 to SKCO1, 1.76–6.14 μM) to resistant (SNUC2B to HCT15, 6.18–47.5 μM), as shown in Figure 1. There is good agreement for the majority of pairs of duplicate cell lines. Thus, COLO201 and COLO206 (4.47 and 4.19 μM), Gp2d and Gp5d (2.54 and 3.80 μM), CACO2 and C2BBe1 (2.50 and 1.42 μM), HDC54 and HDC57 (1.76 and 1.03 μM), and LS174T and LS180 (17.87 and 20.20 μM) agree well with each other. There is a greater difference between DLD1 and HCT15 (18.67 and 47.5 μM), although these two cell lines have the fourth highest and highest GI50 values, respectively, and SW480 and SW620 (6.36 and 17.23 μM), which were established, respectively, from the primary tumour and a metastasis. For all six pairs of these duplicate cell lines, both pairs fall into the same response category. The differences are larger for VAC04A and VAC04S (6.40 and 1.85 μM), which were established from adherent and supernatant subclones of the same cell line. The generally good agreement between lines that have been separately maintained in culture for several years suggests a good measure of reproducibility in the drug response data that we have obtained, and gives confidence to the validity in the categorisation of the cell lines from sensitive to resistant.
concentration of 5FU tested (50 μM): nearly complete inhibition of colony growth at the highest concentration of 5FU tested (50 μM) lies between 0.5 and 5 μM 5FU. This suggests a somewhat higher sensitivity of the cell lines to 5FU in this clonogenicity assay than in our conventional SRB toxicity test, for which GI50 values ranged from 5.76 (SW1222) to 20.2 μM (LS180).

Correlation with genetic data

The classification of the lines into three sensitivity categories for 5FU enables a simple test for association between 5FU response and the genetic changes found in the cell lines. Visual inspection of Figure 1 immediately suggests a strong association between RER+ and 5FU resistance. This is confirmed by the statistical tests, excluding duplicate cell lines, shown in Table 1. The only significant associations among all the investigated parameters in 3 × 2 tables, testing for the association between the three categories of 5FU response and pairs of somatic genetic differences in the cell lines, are for RER+ vs RER− and for LOH around DCC, on chromosome 18. Mutations in TGFbIIR, which are nearly always found in microsatellite unstable tumours because of a coding mononucleotide repeat in the TGFbIIR gene (Markowitz et al., 1995; Samowitz et al., 2002), are on the margin of significance, with P = 0.035. The LOH in SMAD4, also located on chromosome 18, misses significance with P = 0.0759.

The LOH for chromosome 18 and mutations in TGFbIIR are well known to be associated with RER+ vs RER− (Woodford-Richens et al., 2001), as also shown in our data in Table 2. This clearly implies that all these other associations are secondary to that with RER+ vs RER−.

We also found a significant correlation between the d.b.t. of cell lines and their sensitivity to 5FU in a t-test analysis, comparing the average 5FU GI50 differences between the slow and fast (d.b.t. > 48 h and ≤48 h, respectively) growing cell lines (P = 0.0005). Comparing cell lines’ growth rate by RER status also gives a significant result: the average d.b.t. for RER+ lines is 35.7 h, whereas that for RER− lines is longer (48.5 h, P = 0.038, t-test) and this most probably accounts for the d.b.t. association with 5FU response.

DISCUSSION

Our key observation is that 5FU resistance in our panel of 77 CRC cell lines correlates strongly with mismatch repair deficiency, or
These results are in agreement with the clinical data for the adjuvant setting (Ribic et al., 2003; Warusavitarne and Schnitzler, 2007; Muller et al., 2008), and hence support the use of a panel of cell lines for preclinical testing of associations of drug responses with genetic changes in cancers. As a recent meta-analysis by Des Guetz et al. (2009) reported, the situation might be different in metastatic cancer, in which they found an overall hazard ratio (HR) of 0.83 for RER+/RER− patients treated with 5FU-based chemotherapy. It has to be noted, however, that HR ratios varied between 0.48 and 1.21 in the five studies selected by them. When given no adjuvant chemotherapy, RER+ patients have an overall better prognosis (de la Chapelle, 2003; Ribic et al., 2003), as confirmed by a meta-analysis of 32 studies by Popat et al. (2005). This may be explained by immune response to the mutant proteins that arise frequently in RER+ tumours because of frameshift mutations (Bodmer et al., 1994; Saeterdal et al., 2001). This suggestion is supported by the finding that RER+ tumours are characterised by a higher rate of lymphocyte infiltration (Buckowitz et al., 2005) that correlates with the total number of cell lines for preclinical testing of associations of drug responses with genetic changes in cancers. As a recent meta-analysis by Des Guetz et al. (2009) reported, the situation might be different in metastatic cancer, in which they found an overall hazard ratio (HR) of 0.83 for RER+ vs RER− patients treated with 5FU-based chemotherapy. It has to be noted, however, that HR ratios varied between 0.48 and 1.21 in the five studies selected by them. When given no adjuvant chemotherapy, RER+ patients have an overall better prognosis (de la Chapelle, 2003; Ribic et al., 2003), as confirmed by a meta-analysis of 32 studies by Popat et al. (2005). This may be explained by immune response to the mutant proteins that arise frequently in RER+ tumours because of frameshift mutations (Bodmer et al., 1994; Saeterdal et al., 2001). This suggestion is supported by the finding that RER+ tumours are characterised by a higher rate of lymphocyte infiltration (Buckowitz et al., 2005) that correlates with the total number of cell lines for preclinical testing of associations of drug responses with genetic changes in cancers. As a recent meta-analysis by Des Guetz et al. (2009) reported, the situation might be different in metastatic cancer, in which they found an overall hazard ratio (HR) of 0.83 for RER+ vs RER− patients treated with 5FU-based chemotherapy. It has to be noted, however, that HR ratios varied between 0.48 and 1.21 in the five studies selected by them. When given no adjuvant chemotherapy, RER+ patients have an overall better prognosis (de la Chapelle, 2003; Ribic et al., 2003), as confirmed by a meta-analysis of 32 studies by Popat et al. (2005). This may be explained by immune response to the mutant proteins that arise frequently in RER+ tumours because of frameshift mutations (Bodmer et al., 1994; Saeterdal et al., 2001). This suggestion is supported by the finding that RER+ tumours are characterised by a higher rate of lymphocyte infiltration (Buckowitz et al., 2005) that correlates with the total number of...
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The proportion of cell lines in our panel with mutations in MSH6 can be expected to be too low to show any significant correlations. This is also the case for MSH2, in which only three cell lines (LOVO, HCT15, and DLD1) were found to carry MSH2 mutations.

The well-recognised alterations in SMAD4 and DCC in RER+ CRCs and cell lines, as also shown in Table 2, translate into significant differences with respect to 5FU treatment (see Figure 1). This shows further that RER status is the major underlying determinant of 5FU sensitivity.

Following standard doses of 5FU given by intravenous continuous infusion, patient plasma levels reported in the literature lie in the range of 0.06–11.3 μM (188-fold) (Poorter et al, 1995; Aujes et al, 2002), which overlaps the range of GI50 values observed in our cell line panel. This suggests that, under standard therapy, most tumours should have responded to 5FU to some extent, but the patients with RER+ tumours are likely to have responded much less than those with RER− tumours. Such patients should be identified and either given higher doses of 5FU, or not given a drug that is very unlikely to be of benefit for them.

Our data extend significantly previously published data on 5FU responses in cell lines. Thus, studies on a mixed panel of 14 cell lines showed a 31-fold variation in 5FU response (Bracht et al, 2006), when compared with the 1600-fold variation found in our study. Data from the NCI60 drug-screening programme (NSC 199893, 1806 experiments (DTP)) showed variation in GI50 values for 5FU response between 1.6 and 107.6 μM in the limited number of CRC cell lines studied. The most recent previous study on 5FU responses in 30 CRC lines showed a range of GI50 values between 0.7 and 23.1 μM (Mariadason et al, 2003). An analysis of the ranked responses to 5FU in the 21 cell lines included both in Mariadason’s et al (2003) and our study shows a significant correlation, giving P = 0.0307 using the Wilcoxon signed-rank test. These results suggest reasonable reproducibility in the measures of 5FU response even when different techniques are used to assess the response, and lend weight to the use of cell lines for preclinical testing.

Our data agree with a much smaller study on three CRC cell lines undertaken by Carethers et al (1999), who excluded cell cycle alterations and differences in nucleotide uptake as a cause for their findings. The discrepancy between our results and those of Mariadason et al (2003), who did not find RER status correlated with 5FU response, could be explained by differences in the size of the two cell line collections. Although the two panels contain similar proportions of RER+ cell lines (30 and 23.5%) our panel is more than twice the size (30 vs 77 lines with known RER status). We did not detect a correlation between p53 mutation status and 5FU response, which agrees with Mariadason et al (2003), who compared p53 with 5FU apoptotic response.

Although the link between mutations in KRAS and a response to anti-EGFR-based chemotherapy in CRC is well established, the clinical data regarding response to 5FU are more conflicting. Rosty et al (2001) found no significant difference between KRAS mutant and wild-type groups in the response of previously untreated CRC patients with liver metastasis to 5FU. Other researchers report reverse findings, with the 5FU single-agent response rate of 44% in KRAS-mutant patients vs only 32% in wild-type patients (Etienne-Grimaldti et al, 2008). However, the RASCAL study suggested that mutations in KRAS have a negative effect on survival rates and disease relapse (Russo et al, 2005). Our data do not show a significant correlation between KRAS mutation status and 5FU sensitivity (P = 0.2713, data not shown). Analysis of preliminary data on TS mRNA expression and 5FU response suggests that high TS expression may be associated with resistance to 5FU. This has been suggested previously by others (Banerjee et al, 2002).

LS174T cells originate from trypsin treatment and LS180 cells from scraping of the primary culture from a tumour in the same patient (Rutsky et al, 1980). LS180 has been shown to express E-cadherin, whereas Kinsella et al (1994) found LS174T to be nonexpressing. This is explained by LS180 having just one mutation in the E-cadherin gene, whereas LS174T has a second mutation, leading to complete loss of E-cadherin expression in that cell line (Efstathiou et al, 1999 and David Bicknell, unpublished observations). Despite these differences, the cell lines show very similar responses to 5FU treatment, both in the Matrigel colony-forming assay and in SRB-measured response. This suggests that E-cadherin expression, and hence probably epithelial mesenchymal transition (EMT), does not correlate with 5FU response. The overall higher sensitivity of the four cell lines to 5FU in Matrigel compared with our conventional assay is most likely to be because of the difference between a colony-forming assay and a measure of cell number in bulk culture, such as the SRB assay. However, the data do suggest that there is no correlation between 5FU response and the capacity of the cell lines to differentiate. This, together with the lack of difference between LS180 and LS174T, suggests that the cancer stem cells and differentiated cells within the cell lines do not differ substantially in their response to 5FU.

There is now substantial evidence to support the view that cell lines are true representatives of the tumours from which they were derived. Thus, for example, both the frequency of specific mutations as well as their spectrum is similar between cell lines and tumour samples (see, e.g., Douglas et al, 2004; Sanger Institute). In addition, the availability of duplicate cell lines that have been separately maintained in culture for many years, and that in nearly all cases have the same genetic changes, shows that the genetic changes that we studied have not occurred in culture, and hence allows for internal quality control. The use of a large, disease-specific cell panel for drug response and related studies has several advantages: unlimited amounts of material are available, functional studies can be carried out, the size of the panel represents a wide variety of types and stages of CRC, and can help to uncover correlations that would not be observed with a smaller number of cell lines.

We suggest that our approach of studying the response to 5FU in a large panel of well-characterised cell lines, using a relatively high throughput test procedure and an objective categorisation of response, could be a model for preclinical testing of other drugs. Our results show that our approach can be a powerful tool for the detection of predictive markers for drug treatment responses, and that the cell line studies are good predictors of the clinical situation.

ACKNOWLEDGEMENTS

We thank Jenny Wilding for assistance in the discussion of data and preparation of the paper. We also thank all lab members past and present who contributed to the establishment of the database, especially Olympia Dikomitou and David Bicknell. This work was supported in part by a grant from GSK and by a programme grant from CRUK.
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