Microsatellite instability due to hMLH1 deficiency is associated with increased cytotoxicity to irinotecan in human colorectal cancer cell lines

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Around 15% of colorectal cancers (CRCs) show microsatellite instability (MSI) due to dysfunction of the mismatch repair system (MMR). As a consequence of this, MSI tumours tend to accumulate errors in mononucleotide repeats as those in genes implicated in repairing double-strand breaks (DSBs). Previous studies have shown that irinotecan (CPT-11), a chemotherapy agent inducing DSB, is more active in MSI than in microsatellite stable (MSS) CRC. The purpose of this study was to compare the sensitivity to CPT-11 in a series of CRC cell lines with either proficient or deficient MMR and to assess the mutational status of two DSB repair genes, MRE11 and RAD50, in these cell lines. hMLH1-deficient cell lines due to either epigenetic silencing or mutation showed very similar IC50 and were four- to nine-fold more sensitive to CPT-11 than the MSS line. Cell lines harbouring mutations in both MRE11 and RAD50 were most sensitive to CPT-11. We conclude that MSI cell lines display higher sensitivity to CPT-11 than MSS cells. Mutation of MRE11 and RAD50 could account for this difference in response to CPT-11. Future clinical trials tailoring chemotherapy regimens based on microsatellite status are warranted.

Keywords: colorectal cancer; microsatellite instability; RAD50; MRE11; irinotecan

DNA mismatch repair (MMR) proteins correct three types of defects that escape the intrinsic proofreading exonuclease activity of DNA polymerases: (i) single base-pairing errors, (ii) unequal crossing over between microsatellites, and (iii) insertion/deletion loops that result from slippage during replication of repetitive sequences or during recombination. Microsatellites are multiple tandem repeats of a small number of nucleotides that are very prone to these errors; therefore MMR system activity is critical for their maintenance (Kunkel, 2004; Jiricny, 2006). On account of the fact that microsatellites are widely distributed in our genome, mutations of MMR genes affect multiple genetic targets, as those described in mononucleotide repeats of the DNA double-strand breaks (DSBs) repair genes BLM, ATR, DNA-PK, BRCA2, RAD50, and MRE11.

Colorectal cancers (CRCs) are classified as either displaying high-frequency microsatellite instability (MSI-H), low-frequency MSI (MSI-L), or microsatellite stability (MSS) depending on the number of microsatellite loci showing errors by previously defined consensus criteria (Giardiello et al, 2001). Around 15–20% of CRCs are MSI-H, mainly due to epigenetic silencing of the hMLH1 gene promoter (Herman et al, 1998), whereas 2–3% of the total of CRCs are due to germ-line mutations in the MMR genes hMLH1, hMLH2, hMSH6, and PMS2, which are the cause of hereditary non-polyposis CRC (HNPPC) cases (Aaltonen et al, 1998; Salovaara et al, 2000). MSI-H sporadic tumours are characterised by high histologic tumour grade, right-sided location, young age of onset, lower pathological stage, mucinous phenotype with prominent tumour infiltrating lymphocytes, and better prognosis in terms of overall survival than MSI-L/MSS cases (Gryfe et al, 2000; Popat et al, 2005).

CPT-11 is a camptothecin analogue that binds reversibly to DNA topoisomerase I (TOP1) and traps it on the DNA strand, so cleavable complexes will remain stabilised and DNA DSBs will be generated after DNA or RNA polymerases collide with those complexes. This mechanism of action has been named as the ‘fork collision model’ (Pommier, 2006). MMR-deficient CRC tumours and cell lines frequently tend to accumulate mutations within microsatellite repeats of genes implicated in DSB repair pathway (eg, MRE11 and RAD50) (Giannini et al, 2002; Koh et al, 2005), suggesting an enhanced sensitivity of these tumours to camptothecin analogues. In accordance with this fact, emerging clinical data suggest that MSI-H CRC patients may obtain more benefit from CPT-11-based chemotherapy than patients bearing MSS tumours (Fallik et al, 2003; Bertagnolli et al, 2006). Still, preclinical evidence suggesting a higher sensitivity of MMR-deficient tumours...
to irinotecan (CPT-11) is controversial due to discrepant results coming from different studies (Hausner et al, 1999; Jacob et al, 2001; Magrini et al, 2002; Fedier and Fink, 2004).

The objective of this study was to compare the sensitivity to CPT-11 in a series of CRC cell lines classified based on the microsatellite and the mutational status in coding mononucleotide repeats of MRE11 and RAD50. Additionally, we aimed to assess the differences in sensitivity between cell lines with a genetic mutation in MMR genes (MLH1 or MSH6), which resemble HNPCC, and cell lines with silencing of the hMLH1 gene due to the promoter hypermethylation, such as sporadic MSI-H CRC cases.

MATERIALS AND METHODS

Cell lines and culture conditions

HCT-116, SW-48, RKO, and HCT-15 were kindly provided by Dr Manel Esteller (Cancer Epigenetics Laboratory, Spanish National Cancer Centre, Madrid, Spain). HT-29 was obtained from the American Type Culture Collection (Manassas, VA, USA). The microsatellite status of cell lines, the MMR gene mutational status and the analysis of the methylation of hMLH1 promoter was ascertained from the literature and are summarised in Table 1 (Suter et al, 2003). Cells were maintained as monolayers at 37 °C in 5% CO2 air in DMEM: Ham’s F-12 containing 10% foetal bovine serum, glutamine (2 mM), and penicillin/streptomycin (50 IU ml-1).

Western blotting

Cells were grown in 100-mm dishes until subconfluence. After removal of media, cells were washed twice with ice-cold PBS and scraped into ice-cold lysis buffer. After removal of cell debris by centrifugation, protein concentration was determined by Lowry method. Cell samples containing equal amounts of protein were then added to SDS-PAGE loading buffer with 5% mercaptoethanol and heated 5 min at 100 °C. Electrophoretic transfer to nitrocellulose membranes was followed by immunoblotting with the primary antibodies. Finally, membranes were hybridised with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Little Chalfont, UK), and were detected through chemiluminescence with the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA). Rabbit polyclonal antibodies against Mlh-1, Msh-2, and Msh-6 (Upstate, Dundee, UK) were used at 1:1000.

Detection of mononucleotide repeats mutations of MRE11 and RAD50

Mutation analyses for poly(T)11 nucleotide repeats in IVS-4 of MRE11 and poly(A)9 nucleotide repeats in the ORF of RAD50 were performed on genomic DNA extracted using the DNeasy kit (Qiagen, Valencia, CA, USA). PCR conditions and primers were set up as described previously (Kim et al, 2001; Giannini et al, 2002). A 3-μl aliquot of the product was sequenced using the forward and reverse primers with an ABI BigDye TerV3.1 Cycle Sequencing kit on an ABI 9700 thermocycler (Applied Biosystems Inc., Foster City, CA, USA). The data were analysed using Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Cytotoxicity assays and treatment of cultured cells

CPT-11 was obtained from Mayne Pharma Plc (Warwickshire, UK), and it was diluted in DMEM immediately before use. Drug cytotoxicity assays were performed using a modified tetrazolium dye colorimetric assay (cell proliferation reagent WST-1, Roche Applied Science, Penzberg, Germany). Viable cells will metabolise WST-1 to formazan by mitochondrial dehydrogenases and the quantification of formazan dye directly correlates with the number of metabolically active cells that was determined by a scanning microplate reader (ELISA reader). Three thousand cells were seeded per well in 96-well plates and were treated with increasing concentrations of CPT-11 for 48 h (1–100 nmol l-1) after 24 h. After that, cells were washed and incubated in drug-free medium for another 3 days. On day 6, WST-1 was added to each well and further incubated at 37 °C for 1 h. Absorbances were measured and mean values of a minimum of six wells were calculated. Cultures in the absence of drugs were used as positive controls. The percentage of surviving cells at each concentration relative to the non-treated group was plotted and the drug concentrations resulting in 50% of growth inhibition (IC50) were calculated by linear regression analysis of the obtained dose–response curves.

Cell cycle analysis

Cells were fixed with 70% ethanol at –20 °C overnight, followed by treatment with 20 μg ml-1 RNase A and staining with propidium iodide. Fluorescence was measured on a Beckman coulter Epics XL flow cytometer.

Statistical analyses

Differences in CPT-11 sensitivity between cell lines displaying MSI or MSI-L/MSS were analysed using a two-sided t-test with P < 0.05 considered statistically significant. Statistical analyses and linear regression for dose–response curves were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Expression of MMR proteins and mutational status of MRE11 and RAD50

Cell lines were selected to perform the experiments based on their microsatellite status. As shown in Table 1, four cell lines showed MSI. HCT-116 and HCT-15 presented biallelic mutations in hMLH1, hMSH6 genes, respectively, resembling cases of patients

| Cell line | MS status | hMLH1 promoter methylation | hMLH1 | hMSH2 | hMSH6 | RAD50 poly(A)9 exon 13 | MRE11 poly(T)11 intron 4 |
|-----------|-----------|---------------------------|-------|-------|-------|-----------------------|--------------------------|
| HCT-116   | MSI-H     | −                         | mut   | wt    | wt    | −/1 wt                | −/2−1                    |
| HCT-15    | MSI-L     | −                         | wt    | mut   | wt    | 0/0 wt                | 2−2/−1                   |
| SW-48     | MSI-H     | +                         | wt    | wt    | wt    | −/1 wt                | 2−2/−1                   |
| RKO       | MSI-H     | +                         | wt    | wt    | wt    | 0/0 wt                | 2−2/−1                   |
| HT-29     | MSS       | −                         | wt    | wt    | wt    | 0/0 wt                | wt/0 wt                 |

MS = microsatellite; MSI-H = high-frequency microsatellite instability; MSI-L = low-frequency microsatellite instability; MSS = microsatellite stability; mut = mutant; wt = wild type; − = negative; + = positive.
affected by HNPCC. SW-48 and RKO had the hMLH1 gene promoter silenced transcriptionally by hypermethylation as those sporadic tumours showing MSI-H. Contrary, HT-29 displayed normal MMR gene functioning as the majority of sporadic CRC cases. Expression of hMLH1, hMSH2, and hMSH6 were ascertained by western blot (Figure 1). All CRC cell lines used in our study expressed some level of Msh2. The expression of Mlh1 was undetectable in HCT-116, SW-48, and RKO, whereas Msh6 protein was detected in all cell lines except for HCT-15, corresponding to the known mutational status.

We have studied the presence of mutations in microsatellite regions of MRE11 and RAD50 that are involved in surveillance and correction of DSBs, as those generated after treatment with CPT-11. In particular, we have investigated the occurrence of a frameshift mutation in a coding poly(A) repeat of RAD50 and a shortening in an intronic poly(T) repeat of MRE11. These mutations result in a truncated protein in the case of RAD50 and an aberrant transcript due to skipping of exon 5 and a premature stop codon introduction in MRE11. We showed that all MSI cell lines harboured mutations in the poly(T) tract of MRE11 (Table 1). In addition, HCT-116 and RKO presented mutations in the poly(A) of RAD50. The MMR-proficient cell line HT-29 was wild type for both genes.

**Cytotoxicity of CPT-11 to CRC cell lines classified by microsatellite status**

Dose–response curves were plotted to determine the IC50 and 95% confidence intervals for each cell line (Figure 2). As shown in Figure 3, hMLH1-deficient cell lines due to either epigenetic silencing (RKO) or biallelic mutation (HCT-116) were the most sensitive to CPT-11 in comparison to the MMR-proficient HT-29, which had an IC50 four- to nine-fold higher (MSI-H vs MSI-L/MSS, *P* < 0.001). In addition, both cell lines presenting hypermethylation of hMLH1 had a similar IC50 showing consistency in our results. However, the hMSH6-deficient cell line, HCT-15, displayed sensitivity to CPT-11 closer to the MSS cell line than the rest of MSI cell lines, suggesting a minor role of hMSH6 deficiency in the sensitivity to CPT-11.

Regarding the mutational status of DSB repair genes and response to CPT-11, we have observed that HCT-116 and RKO, which harboured mutations in both MRE11 and RAD50, were the most sensitive cell lines. On the contrary, HT-29 was wild type for both genes and was most resistant to CPT-11. Noteworthy, HCT-15 was heterozygous for the poly(T) mutation in MRE11 and wild type for RAD50 and was less sensitive to CPT-11 than HCT-116 and RKO. Therefore, the presence of mutations in DSB repair genes may explain the differences in sensitivity between MSI and MSS cells, whereas the number of affected DSB repair genes may account for different levels of sensitivity to CPT-11.

**Figure 1** Expression of hMSH6 (160 kDa), hMSH2 (100 kDa), hMLH1 (83 kDa), and β-actin (42 kDa) were analysed by western blotting in whole-cell protein extracts.

**Figure 2** Cytotoxicity of CPT-11 to human colorectal cancer cell lines.

**Figure 3** Cytotoxicity to CPT-11 of CRC cell lines. Histograms represent the IC50 and error bars represent 95% CI. IC50 concentrations resulting in 50% of growth inhibition; 95% CI, 95% confidence intervals.

**Cell cycle analysis after treatment with different concentrations of CPT-11**

We did perform cell cycle analysis based on DNA content after exposure to two different concentrations of CPT-11 for 48h. We selected 5 and 10 μmol/l because the former is around the IC25 in the most sensitive cells (MMR deficient) and the latter is around the IC50 in the least sensitive (MMR proficient). Moreover, these concentrations are similar to peak plasma concentrations after CPT-11 infusion in human subjects (Mathijssen et al, 2001). As shown in Figure 4, the percentage of cells in S phase without treatment was 14.7–39.6%, and in G2 phase was 14.7–27.1%. Treatment with CPT-11 decreased the percentage of cells in S phase and elicited an arrest in the G2/M phase of cell cycle. When we compared the changes in the percentage of cells in S, G1 and...
Figure 4 Exposure to CPT-11 for 48 h results in G2/M arrest. CRC cell lines were treated with 5 μM to 10 μM CPT-11, and supplemented medium (control). Cell cycle analyses were performed by FACS.

However, sensitivity of CPT-11 in this subset of tumours is not so clearly defined at both the preclinical and the clinical levels. Two independent research groups have reported resistance to camptothesins in MMR-deficient cell lines (Hauser et al., 1999; Fedier et al., 2001). In contrast with these reports, we and others (Jacob et al., 2001; Magrini et al., 2002) have demonstrated higher sensitivity to CPT-11 in MMR-deficient human CRC cell lines than in MMR-proficient cells and G2/M arrest after exposure to CPT-11. Likewise, the body of evidence supporting the sensitivity to CPT-11 in MSI-H CRC at the clinical level is limited. A retrospective analysis of 73 metastatic CRC patients who received second-line CPT-11-based chemotherapy showed a higher response rate in the group of tumours displaying MSI-H compared with the MSI-L/MSS group (57.1% vs 10.8%, P-value = 0.009) (Fallik et al., 2003). Bertagnolli et al. (2006) analysed the differences in disease-free survival in 482 patients enrolled in the CALGB 89803 adjuvant study according to the microsatellite status. A total of 1264 patients with resected stage III colon cancer were randomized to receive adjuvant 5-FU-based treatment with or without CPT-11. Sixteen percent of patients had MSI-H tumours. Regarding the population receiving CPT-11 in addition to 5-FU, those patients bearing MSI-H tumours presented a higher disease-free survival than those patients with MSI-L/MSS tumours. Despite this, improvement in survival did not achieve statistical significance, although there was a clear trend for a prolongation in disease-free survival (long rank = 0.18). Several reasons may explain the lack of significant differences found in this study. First, the results may not be mature enough due to the limited follow-up of the patients (mean follow-up = 3.8 years). Second, the retrospective nature of this analysis along with the restriction to only one-third of the patients included in the clinical study may either be generating an uncontrolled bias or limiting the power to detect differences. Finally, adjuvant CPT-11-based chemotherapy has globally failed to demonstrate a significant improvement in terms of progression-free survival in stage III CRC in three different studies (van Cutsem et al., 2005; Ychou et al., 2005; Saltz et al., 2007), which suggests that factors other than MSI status play an important role as prognostic factors in the adjuvant setting.

The current evidence in the treatment of advanced CRC patients shows that multitarget chemotherapy incorporating either oxaliplatin (de Gramont et al., 2000; Giacchetti et al., 2000) or CPT-11 (Douillard et al., 2000; Saltz et al., 2000) onto a backbone of...
fluoropyrimidines is superior to traditional regimens of fluoropyrimidines alone (Kelly and Goldberg, 2005). However, the available randomised studies have failed to demonstrate that a first-line oxaliplatin combined with infusional 5-FU schedule, or a CPT-11 with infusional 5-FU schedule, was better than the other for the global population of patients with advanced CRC (Giacchetti et al, 2000; Tournigand et al, 2004). Further exploration on the role of microsatellite status to select the most appropriate drug combination for the first-line treatment of metastatic CRC may be considered in the design of future clinical trials.

One of the questions that still remains partially unanswered is the exact mechanism why MSI-H cells are more sensitive to camptothecins than MSS cells. Several studies have demonstrated that deficiency in the MMR genes leads to secondary mutations in microsatellite tracts as those present in the mononucleotide repeats regions of the principal caretaker genes implicated in the repair of DSB, such as the Mre11/Nbs1/Rad50 protein complex (Giannini et al, 2004; Koh et al, 2005). These observations have been available randomised trials have failed to demonstrate that pyrimidines alone (Kelly and Goldberg, 2005). However, the fluoropyrimidines is superior to traditional regimens of fluoropyrimidines alone (Kelly and Goldberg, 2005). However, the available randomised studies have failed to demonstrate that a first-line oxaliplatin combined with infusional 5-FU schedule, or a CPT-11 with infusional 5-FU schedule, was better than the other for the global population of patients with advanced CRC (Giacchetti et al, 2000; Tournigand et al, 2004). Further exploration on the role of microsatellite status to select the most appropriate drug combination for the first-line treatment of metastatic CRC may be considered in the design of future clinical trials.

In conclusion, our study reinforces previous preclinical results suggesting an increased sensitivity to CPT-11 in MSI-H CRC cell lines. This chemosensitivity is explained by an impairment of the mechanism to repair DSB caused by DNA-damaging agents secondary to the microsatellite instability. In addition, we have demonstrated that no differences in sensitivity exist between cell lines with inactivation of the Mlh-1 function secondary to biallelic mutations and those with epigenetic silencing of the promoter. These results together with the available clinical information support the design of more clinical trials with CPT-11-based chemotheraphy regimens in the adjuvant and advanced settings according to a tailored approach that combines clinical characteristics and the MSI status as a predictive factor of response to chemotherapy.

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