Chapter 6

DCR1 promoter hypermethylation and response to irinotecan in metastatic colorectal cancer.

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In preparation.
Diversity in the biology of colorectal cancer (CRC) is associated with variable responses to standard chemotherapy. We aimed to identify and validate DNA hypermethylated genes as predictive biomarkers for irinotecan treatment of patients with metastatic CRC.

Candidate genes were selected from 389 genes involved in DNA Damage Repair by correlation analyses between gene methylation status and drug response in 32 cell lines. The discovery and initial validation set consisted of primary tumors of 185 and 166 metastatic CRC patients, respectively, from the phase III CAIRO trial. An external validation set consisted of 467 primary tumors from the phase III FOCUS study. Methylation status in tumor tissue was correlated to progression free survival (PFS) by first-line treatment regimen, containing either single-agent fluorouracil (i.e. CAP in CAIRO or 5FU in FOCUS) or combination chemotherapy (i.e. CAP or 5FU plus irinotecan (CAPIRI in CAIRO / FOLFIRI in FOCUS)).

In the discovery and initial validation set, patients with methylated DCR1 tumors did not significantly benefit from CAPIRI treatment over CAP treatment (discovery set: HR=1.2 (95%CI 0.7-1.9, \(p=0.6\)), validation set: HR=0.9 (95%CI 0.6-1.4, \(p=0.5\))), whereas patients with unmethylated DCR1 tumors did (discovery set: HR=0.4 (95%CI 0.3-0.6, \(p=0.0001\)), validation set: HR=0.5 (95%CI 0.3-0.7, \(p=0.0008\))). These results, however, could not be validated in the external data set, where a similar effect size was found in patients with methylated and unmethylated DCR1 (methylated DCR1: HR=0.7 (95%CI 0.5-0.9, \(p=0.01\)), unmethylated DCR1: HR=0.8 (95%CI 0.6-1.2, \(p=0.4\))).

DCR1 promoter methylation was identified and initially validated as a potential negative predictive biomarker for response to irinotecan-based therapy, but external validation could not validate these findings. These results underline the importance of extensive clinical evaluation of candidate biomarkers.
INTRODUCTION

The outcome of patients with colorectal cancer (CRC) strongly depends on tumor stage at time of diagnosis. Whereas stage I CRC patients have a 5-year overall survival of more than 90%, in stage IV CRC patients it declines to ~20% or less(1). When distant irresectable metastases develop, palliative systemic therapy is the only treatment option available to these patients. The backbone of this is 5-fluorouracil (5FU) in combination with either oxaliplatin or irinotecan(2). More recently, addition of targeted agents directed against vascular epithelial growth factor (VEGF) (bevacizumab) or epidermal growth factor receptor (EGFR) (cetuximab and panitumumab) has been demonstrated to give additional survival benefit (3). Only a subset of patients benefit from these regimens, while those patients that do not, still may suffer from considerable toxicity. With the exception of KRAS mutation status that predicts resistance to EGFR-targeted therapy(4-6), no other biomarkers exist that adequately predict treatment response in metastatic CRC. Thus, predictive biomarkers are urgently needed to a priori identify the subset of patients that will benefit from a specific treatment.

Hypermethylated genes form a particular category of biomarkers and a number of these have been reported to predict drug response in CRC patients(7, 8), but inconsistent results for the same markers have been reported(9, 10). Hypermethylated genes are of particular interest, since DNA methylation is potentially reversible by DNA methyltransferase inhibitors, which could provide a way to restore expression of genes silenced by DNA hypermethylation and thus increase the sensitivity of tumor cells to the agents the gene is associated with(11, 12).

In the present study we set out to identify and validate novel hypermethylated genes that predict response to treatment with irinotecan in patients with metastatic CRC, using material from two clinical trials, i.e. the Dutch CApecitabine, IRinotecan and Oxaliplatin (CAIRO) study(13) and the Fluorouracil, Oxaliplatin, CPT-11: Use and Sequencing (FOCUS) study from the UK(14).

MATERIALS AND METHODS

Candidate gene selection

Candidate gene selection was based on correlations between gene methylation and drug response in cell lines, which is described in detail in the Supplementary Information. In short, the methylation status of 389 genes involved in DNA Damage Repair and Response was determined in 32 cell lines of different tissue origin, and correlated with the sensitivity to 118 drugs in these same cell lines as published by The Genomics and Bioinformatics Group(15).

(http://discover.nci.nih.gov/nature2000/data/selected_data/a_matrix118.txt). The sensitivity (i.e. -log(GI50)) scores to 118 drugs were translated into 15 common modes of action. For the present study that focused on irinotecan sensitivity we selected genes, methylation of which was associated with topoisomerase inhibitors-related mode of action.
Patient sample selection

Patients selected for the current study participated in either of two phase III trials, namely the CApecitabine, Irinotecan and Oxaliplatin (CAIRO) study of the Dutch Colorectal Cancer Group (DCCG) (CKTO 2002-07, ClinicalTrials.gov; NCT00312000)(13), and the Medical Research Council Fluorouracil, Oxaliplatin, CPT-11: Use and Sequencing (FOCUS) study (ISRCTN 79877428) under the auspices of the United Kingdom National Cancer Research Institute Colorectal Cancer Studies Group(14). Written informed consent was required from all patients before study entry, and included consent for translational research on tumor tissue. In the CAIRO study, 820 patients with metastatic CRC without prior palliative systemic treatment were randomized between sequential (arm A; first-line capecitabine (CAP), second-line irinotecan and third-line capecitabine plus oxaliplatin (CAPOX)) and combination treatment (arm B; first-line capecitabine plus irinotecan (CAPIRI) and second-line CAPOX). In the FOCUS study, 2135 metastasized CRC patients without prior palliative systemic treatment were randomly assigned to three treatment strategies in the ratio 1:1:1. Strategy A (control group) was single-agent 5FU until failure, then single-agent irinotecan. Strategy B was 5FU until failure, then randomly assignment to 5FU plus irinotecan (B-ir; FOLFIRI) or 5FU plus oxaliplatin (B-ox; FOLFOX). Strategy C was random assignment to FOLFIRI (C-ir) or FOLFOX (C-ox) from the outset. Separate consent was requested for access to stored histopathological specimens. Multicenter ethical approval was obtained. The trial was managed by the Medical Research Council Clinical Trials Unit, overseen by an independent trial steering committee which also prospectively approved the molecular analysis plan.

The comparison of CAPIRI versus CAP (CAIRO) and FOLFIRI vs 5FU (FOCUS) as first line therapy provided an excellent opportunity to evaluate predictive markers for irinotecan in the current study.

CAIRO biomarker populations

An initial 185 patients were selected for a discovery set of which 90 patients were treated with first-line CAP and 95 were treated with first-line CAPIRI. The patient samples were matched according to the stratification factors in the original study (for the subgroup of patients that underwent resection of the primary tumor, since these are the patients from whom material was available to be included in this study)(13), that is, performance status, predominant metastatic site, previous adjuvant therapy and serum lactate dehydrogenase level (LDH). In addition, only patients were included who had received at least 3 cycles of 1st line therapy, or 2 cycles when death followed due to progressive disease. A large proportion of these samples overlap with samples described in (16).

For the initial validation set, patients who had received at least 3 cycles of 1st line therapy or 2 cycles when death followed due to progressive disease were selected, with no further criteria, from the remaining patients of which tumor DNA samples were available. These concerned 166 patients, of which 78 were treated with first-line CAP and 88 were treated with first-line CAPIRI.
**FOCUS biomarker validation population**

A total of 467 tumor DNA samples from the FOCUS trial were available for the current study. These came from 331 patients treated with at least 3 cycles of first-line 5FU (249 from strategy A and 82 from strategy B-ir) and 136 patients treated with at least 3 cycles of first-line irinotecan (all from strategy C-ir).

**DNA isolation and methylation analysis**

Tumor samples from the CAIRO trial were retrieved through the Dutch national pathology registry PALGA(17). From formalin-fixed paraffin-embedded tissue samples from primary tumors, resected before chemotherapy, DNA was extracted as described before(18, 19). DNA concentrations were quantified using the Nanodrop 1000 UV spectrophotometer (Nanodrop Technologies Inc, Wilmington, DE, USA). DNA was subjected to sodium bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer’s protocol.

Tumor DNA from the FOCUS trial was extracted as described in (20). DNA samples were subsequently cleaned by ethanol precipitation and DNA concentrations were quantified using the Nanodrop 1000 UV spectrophotometer (Nanodrop Technologies Inc).

All methylation assays were performed blindly to information on treatment or survival outcome. The CAIRO discovery set was subjected to high-throughput LightCycler MSP assay (LightCycler 480 SYBR Green I Master kit (Roche, Vilvoorde, Belgium)) for the 22 selected candidate genes. Primers were designed in promoter regions (i.e. -1000 to +200 bp relative to the transcription start site). Primers from literature were used when they experimentally passed our quality control; see supplementary table 1 for primer sequences. Quality control was performed with in vitro Methylated DNA (Chemicon, Temecula, CA) as a positive control and DNA from the unmethylated human HCT116 DKO cell line as a negative control. Per sample, 20 ng bisulfite-modified DNA was amplified with the following PCR conditions: 95°C for 10 minutes followed by 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 1 second. Amplification of beta-actin was used as an unmethylated reference gene. The amplicons were checked for size and quantified by capillary electrophoresis (LC90 Labchip; Caliper Lifesciences).

For the CRC cell lines and the CAIRO validation set a quantitative MSP (qMSP) assay for DCR1 was used. The primers for methylated DNA were equal to the primers used for LightCycler analyses described above and were designed at the exact location as described before(21). qMSP reactions were carried out in duplicate in 25 µl reaction volumes, each containing 36 ng of bisulfite-treated DNA, 10 pmol of each primer and 1x Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Each plate included no template controls and a standard curve with a serial dilution of bisulfite-modified DNA from a mixture of methylated cell line (HCT116) and unmethylated cell line (HCT116-DKO). PCR conditions were 95°C for 15 minutes, followed by 40 cycles at 95°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds, followed by a melt curve stage to check the specificity of the amplification reaction. Cycle threshold (Ct) values were measured at a fixed
fluorescence threshold, which was always in the exponential phase of the amplification curves. The methylation percentage per sample was calculated according to the formula $2e^{-[\text{mean Ct}^{\text{M-reaction}}]/(2e^{-[\text{mean Ct}^{\text{U-reaction}}]}+2e^{-[\text{mean Ct}^{\text{U-reaction}}]}) \times 100$. The U (unmethylated) and M (methylated) reactions were amplified with comparable efficiencies. A sample was called methylated when the methylation percentage was higher than observed in a panel of 21 normal colon mucosa from non-cancer patients as measured in triplicate (median plus 2 times the standard deviation = 6%).

The FOCUS validation set was analyzed with a qMSP assay for DCR1 as well. The primers for methylated DNA were equal to the primers used in the CAIRO discovery and validation study. qMSP reactions were carried out using a 7500 Fast Real-Time PCR System (Applied Biosystem,) in duplicate in 25 µl reaction volumes, where each reaction contained 40 ng of bisulfite-treated DNA, 10 pmol of each primer and 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems). Each plate included no-template controls and a standard curve with a serial dilution of bisulfite-modified DNA prepared from in vitro Methylated DNA (Chemicon). PCR conditions were 95°C for 15 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 60 seconds, followed by melt curve analysis to check the specificity of the amplification reaction. Amplification of beta-actin was used as an unmethylated reference gene, using the same PCR conditions. The Ct ratio per sample was calculated according to the formula $2e^{-[\text{mean Ct}^{\text{DCR1}}] - \text{mean Ct}^{\text{ACTB}}}$. A sample was called methylated when the Ct ratio was higher than observed in a panel of 22 normal colon mucosa from non-cancer patients as measured in duplicate (median plus 2 times the standard deviation = 0.006).

**Cell lines**

HCT15, HCT116, LS513, LS174T, Colo320, SW48, SW1398, HT29, Colo205, SW480, and RKO were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Lonza Biowhittaker, Verviers, Belgium) containing 10% fetal bovine serum (Hyclone, Perbio, UK). Caco-2 was cultured in RPMI 1640 (Lonza Biowhittaker) containing 20% fetal bovine serum. LIM1863 was cultured in RPMI 1640 (Lonza Biowhittaker) containing 5% FCS, 0.01 mg/ml thioglycerol, 1 mg/ml insulin and 1 µg/ml hydrocortisone. All cell culture media were supplemented with 2 mM L-glutamine, 100 IU/ml sodium penicillin (Astellas Pharma B.V., Leiderdorp, The Netherlands) and 100 mg/ml streptomycin (Fisiopharma, Palomonta (SA), Italy). To investigate re-expression of DCR1 after inhibition of DNA methyltransferases, HCT116 and Colo205 cells were treated with 5000 nM 5-aza-2’-deoxycytidine for 3 days (DAC, Sigma Chemical Co., St. Louis, MO, USA).

**RNA isolation and qRT-PCR**

Total RNA was isolated from cell lines using Trizol reagent (Invitrogen, Breda, the Netherlands), and subjected to purification using RNeasy Mini Kit (Qiagen). After DNAse treatment (RQ1 DNAse, Promega, Leiden, the Netherlands), cDNA was made with the Iscript cDNA Synthesis Kit (BioRad, Veenendaal, the Netherlands). Quantitative RT-PCR was done using TaqMan® Gene Expression Assays
from Applied Biosystems directed to \textit{DCR1} (Hs00182570\_m1) and \textit{B2M} (Hs00984230\_m1). Relative expression levels were determined by calculating the Ct-ratio (Ct ratio = $2e^\left(-\left(Ct_{DCR1}-Ct_{B2M}\right)\right)\times1000$).

**TCGA data**

\textit{DCR1} DNA methylation (Illumina Infinium HM27 bead array; HM27) and mRNA expression (Agilent array) data were obtained via cBioPortal for Cancer Genomics (http://www.cbioportal.org; (22)) on 223 CRC tumors included in The Cancer Genome Atlas (TCGA) Colorectal Cancer project. This data set was downloaded on the 14th of July 2015 from all tumors with available methylation and mRNA expression data from the Colorectal Adenocarcinoma (TCGA, Nature 2012) dataset (23).

**Statistical analysis**

The primary endpoint of the present study was progression free survival (PFS) under first-line systemic therapy with or without irinotecan. PFS for first-line treatment was calculated from the date of randomization to the first observation of disease progression or death reported after first-line treatment. To test the predictive value of candidate genes, multivariate Cox proportional hazard models were built that included the variables treatment, candidate gene and an interaction term treatment*candidate gene. In the CAIRO as well as the FOCUS trial, patients were randomized between treatment arms, which resulted in similar clinical characteristics between the treatment arms. However, because we were analyzing patient subsets from the original trials, we corrected the estimates of predictive value of candidate genes for those variables that could have possible prognostic effect, by including them in the multivariate analyses. These were age, gender, WHO performance status and prior adjuvant therapy for both the CAIRO and the FOCUS samples, plus normal or abnormal LDH and location of metastases for CAIRO. Cox proportional hazard models were used to estimate Hazard Ratios (HR) and 95\% confidence intervals (CI). Kaplan-Meier analyses and log-rank tests were used to estimate survival over time. Correction for multiple testing in the discovery set was done by the Benjamini Hochberg method.

Student’s T-test was used for comparison of \textit{DCR1} expression levels before and after DAC treatment of HCT116. Pearson correlation analysis was used to measure correlation between \textit{DCR1} methylation and mRNA expression levels from 223 primary CRC tissue samples as provided by The Cancer Genome Atlas (TCGA) database.

Statistical analyses were performed using the computing environment R version 3.2.(24), including the packages \textit{survival} and \textit{rms}(25-27).
RESULTS

Candidate gene selection

Correlation analyses of the DNA methylation status of 389 genes involved in DNA Damage Repair and Response with sensitivity to 118 drugs in 32 cell lines yielded 22 genes associated with topoisomerase-inhibitor related mode of action. These genes were analyzed for DNA methylation status in the discovery set (n=185). Methylation frequencies ranged from 5% to 98%, average 43% (supplementary table 2).

Evaluation of biomarker potential in the discovery set (CAIRO)

In the 185 patients selected for the discovery set, we first evaluated whether the methylation status of the selected candidate genes had any prognostic impact on PFS, independent of the given treatment. None of the 22 candidate genes showed an association between methylation status and outcome in terms of PFS (supplementary table 2).

Next, we evaluated whether the methylation status of the candidate genes had a predictive value in relation to treatment with CAPIRI. In concordance with the original CAIRO study (13), the patients of the discovery set showed significantly longer PFS when treated with CAPIRI (n=95) compared to CAP alone (n=90) (median PFS of 252 vs 182 days for CAPIRI vs CAP, respectively; HR=0.67 (95% CI 0.50-0.90, p=0.007) (figure 1A). To assess the predictive value of each candidate gene, a multivariate survival model was made. The model included clinical variables (age, gender, normal or abnormal LDH, WHO performance status, prior adjuvant therapy and location of metastases), treatment arm and an interaction term between treatment arm and candidate gene. After correcting for multiple testing, the treatment arm*candidate gene interaction remained significant for DCR1 and IRAK1. This indicates that the methylation status of these candidate genes exerted an independent effect on PFS that was different in the one treatment arm compared to the other treatment arm (table 1).

Kaplan-Meyer curve analysis revealed that out of the two final candidate genes, the methylation status of DCR1 was predictive of PFS after treatment with CAPIRI, but not for PFS after treatment with CAP; patients with methylated DCR1 tumors progressed more quickly than patients with unmethylated DCR1 tumors when treated with CAPIRI (HR=2.1 (95% CI 1.3-3.3, p=0.001), but no difference was observed between patients with unmethylated or methylated DCR1 tumors when treated with CAP (HR=0.7 (95% CI 0.5-1.1, p=0.1) (supplementary figure 1). IRAK1 methylation was predictive of PFS after treatment with CAP and hence was not further studied (supplementary figure 1).

Because CAIRO was a randomized controlled trial, we were able to estimate the benefit of CAPIRI treatment over CAP treatment for patients with methylated or unmethylated DCR1 tumors by comparing PFS between the different treatment arms. Patients with methylated DCR1 (72 out of 185; 39%) did not benefit from adding irinotecan to CAP (median PFS of 192 vs 184 days for CAPIRI vs CAP, respectively; HR=1.2 (95%CI 0.7-1.9, p=0.6; figure 1B)). In contrast, patients with unmethylated
DCR1 showed a significantly longer PFS when treated with CAPIRI compared to CAP alone (median PFS of 270 vs 178 days for CAPIRI vs CAP, respectively; HR=0.4 (95% CI 0.3-0.6, p=0.00001; figure 1C)).

**Internal validation set (CAIRO)**

In order to validate methylated DCR1 as a marker for lack of response to irinotecan-based therapy, a second set of patients from the CAIRO study was examined. Also in this series, in concordance with the original CAIRO study (13), PFS was significantly longer for patients treated with CAPIRI (n=88)
Figure 1. CAIRO discovery set: Progression-free survival in metastatic CRC cancer patients treated in first-line with CAP (blue line) or CAPIRI (red line) in (A) all patients from the CAIRO discovery set, in (B) patients with methylated tumor DCR1 or in (C) patients with unmethylated tumor DCR1.

95% confidence interval of the survival probability is shown by blue and red shades. HR=Hazard Ratio (CAPIRI versus CAP).
FIGURE 2. CAIRO validation set: Progression-free survival in metastatic CRC cancer patients treated in first-line with CAP (blue line) or CAPIRI (red line) in (A) all patients from the CAIRO validation set, in (B) patients with methylated tumor DCR1 or in (C) patients with unmethylated tumor DCR1.

95% confidence interval of the survival probability is shown by blue and red shades. HR=Hazard Ratio (CAPIRI versus CAP).

A

CAIRO validation - all patients
CAPIRI vs CAP:
Median PFS 267 vs 200 days
HR = 0.6
p = 0.003

Nr at risk:
CAP 78 39 8 4 3
CAPIRI 88 63 23 8 4

B

CAIRO validation - DCR1 methylated
CAPIRI vs CAP:
Median PFS 267 vs 203 days
HR = 0.9
p = 0.5

Nr at risk:
DCR1 U 42 21 6 3 2
DCR1 M 46 32 8 3 1

C

CAIRO validation - DCR1 unmethylated
CAPIRI vs CAP:
Median PFS 261 vs 195 days
HR = 0.5
p = 0.0008

Nr at risk:
DCR1 U 36 18 2 1 1
DCR1 M 42 31 15 5 3
compared to patients treated with CAP alone (n=78) (median PFS of 267 vs 200 days for CAPIRI vs CAP, respectively; HR=0.6 (95% CI 0.5-0.9, p=0.003; figure 2A)).

DCR1 was methylated in 88 out of 166 (53%) tumors. A multivariate analysis, as described for the discovery set, showed a significant interaction between treatment arm and DCR1 methylation (p=0.04, table 2). Kaplan-Meyer analyses confirmed that patients with methylated DCR1 tumors did not significantly benefit from CAPIRI treatment over CAP treatment (median PFS of 267 vs 203 days for CAPIRI vs CAP, respectively; HR=0.9 (95%CI 0.6-1.4, p=0.5; figure 2B)), whereas patients with unmethylated DCR1 tumors did (median PFS of 261 vs 195 days for CAPIRI vs CAP, respectively; HR=0.5 (95%CI 0.3-0.7, p=0.0008) (figure 2C).

### Table 2. Evaluation of predictive value of DCR1 methylation on progression after treatment (multivariate cox proportional hazard model).

<table>
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<th>CAIRO validation set</th>
<th>FOCUS validation set</th>
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HR = Hazard Ratio

**External validation set (FOCUS)**

As an independent validation of DCR1 methylation as a marker for response to irinotecan-based therapy, we tested a large series of tumor samples from another randomized controlled phase III clinical trial (FOCUS) (14). From this cohort, we selected patients that received a combination of fluorouracil plus irinotecan (FOLFIRI) or fluorouracil (5FU) alone as first-line therapy. A total of 467 tumor samples were analyzed. In this series, similar to the CAIRO trial (13) and the original FOCUS trial (14), PFS was significantly longer for patients treated with FOLFIRI (n=136) compared to patients treated with 5FU alone (n=331) (median PFS of 272 vs 231 days for CAPIRI vs CAP, respectively; HR=0.8 (95%CI 0.6-1.0, p=0.02); figure 3A).

DCR1 was methylated in 225 out of 467 (48%) tumors. DCR1 methylation status was tested multivariately for association with PFS together with the variables treatment arm and an interaction term between treatment arm and candidate gene. Clinical characteristics (age, gender, WHO performance status and prior adjuvant therapy) were included in the model as well. No significant
**FIGURE 3.** FOCUS validation set: Progression-free survival in metastatic CRC cancer patients treated in first-line with 5FU (blue line) or FOLFIRI (red line) in (A) all patients from the FOCUS validation set, in (B) patients with methylated tumor DCR1 or in (C) patients with unmethylated tumor DCR1.

95% confidence interval of the survival probability is shown by blue and red shades. HR=Hazard Ratio (FOLFIRI versus 5FU).
interaction between treatment arm and DCR1 methylation status was observed ($p=0.3$, table 2). Indeed, Kaplan-Meyer analyses, stratified for presence of absence of methylated DCR1, revealed that patients with methylated or unmethylated DCR1 had a similar effect size from FOLFIRI treatment.

**FIGURE 4. DCR1 methylation and mRNA expression levels.** A. DCR1 mRNA expression analysis in CRC cell lines by RT-PCR. DCR1 DNA methylation percentage as measured by qMSP (M%) is indicated below each cell line. Quantifications represent mean expression values from three independent experiments. B. DCR1 mRNA expression analysis by RT-PCR of HCT116 (left panel) and Colo205 (right panel) with and without DAC treatment ($p=0.005$ and $p=0.08$, respectively). C. Scatter plot including a linear regression line and 95% confidence interval, showing the correlation of DCR1 methylation levels and DCR1 mRNA expression in 223 CRC tissues from TCGA.
over 5FU treatment, which reached significance for patients with methylated DCR1 (methylated DCR1: median PFS of 283 vs 225 days for FOLFIRI vs 5FU, respectively; HR=0.7 (95%CI 0.5-0.9, p=0.01); figure 3C; unmethylated DCR1: median PFS of 253 vs 235 days for FOLFIRI vs 5FU, respectively; HR=0.8 (95%CI 0.6-1.2, p=0.4) (figure 3B)).

**Methylation of DCR1 is associated to decreased gene expression**

A lack of correlation between DCR1 methylation and DCR1 gene expression could be one of the reasons why we were not able validate DCR1 methylation as marker for response to irinotecan-based therapy. We therefore tested whether the observed DNA methylation was associated with its gene expression. This was investigated *in vitro* in a panel of 13 CRC cell lines. Ten out of 13 CRC cell lines were fully methylated for DCR1 and showed low or absent gene expression. The other three CRC cell lines were hemi-methylated and showed clearly higher gene expression levels (figure 4A). Treatment of two CRC cell lines, HCT116 and Colo205, with the demethylating agent DAC resulted in increased DCR1 expression (p=0.005 and p=0.08, respectively; figure 4B). In addition, data from The Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov), including 223 CRC tumors, confirmed a negative correlation between DCR1 DNA methylation and DCR1 mRNA expression (Pearson correlation of -0.4, p=3.4E-9; figure 4B).

**DISCUSSION**

CRC is not only clinically but also biologically a heterogeneous disease. Much of the biological diversity is initially defined at the DNA level (mutations, copy number changes and promoter hyper-methylation) and subsequently propagated at the RNA and protein level, giving rise to phenotypical differences and differences in clinical behavior, including risk of metastasis and response to systemic treatment. While most of the drugs available for CRC are used as “one size fits all”, it is evident from their different modes of action that differences in biology may affect response to these drugs.

In the present study we used a candidate gene approach to identify methylation markers for response to treatment with irinotecan-based therapy. We first made a selection of candidate genes based on *in vitro* findings on their function in relation to the mode of action of irinotecan, i.e. topoisomerase inhibition. We next tested for correlation of the methylation status of the candidate genes and PFS after treatment with CAPIRI therapy of metastatic CRC patients participating to the phase III CAIRO trial(13), which identified DCR1 as a candidate marker. Because patients treated with CAP alone were used as a control group, this analysis showed DCR1 methylation as a potential negative predictive marker for response to irinotecan-based therapy. The initial finding in the discovery set could be confirmed in a second series of patients from the same CAIRO study, which indicated that the initial finding was not a stochastic statistical finding. In addition, analysis on sixty-five patients in the CAIRO sequential treatment arm who were analyzed for their response to single agent irinotecan therapy in second line, showed that patients with methylated DCR1 tumors yielded a shorter PFS.
compared to patients with an unmethylated DCR1, although this difference did not reach statistical significance (data not shown). However, validation in a second, independent series of metastatic CRC patients from the phase III FOCUS trial(14), treated with first-line FOLFIRI or 5FU alone, did not confirm the negative predictive value of DCR1 methylation status to irinotecan-based therapy.

Developing predictive biomarkers that reach the phase of introduction into clinical practice has proven to be highly challenging. Literature is full of proof of concept publications on potential biomarkers, but in most instances no further validation follows. The current study was carefully designed in order to overcome most common pitfalls in biomarker discovery (28, 29); i.e. a strong biological rationale existed for the preselected candidate genes, and extensive evaluation (discovery, internal validation and external validation) was performed in a prospective-retrospective design(30) on in total 818 archival tumor samples derived from two similar well-conducted phase III randomized clinical trials, providing the highest quality of clinical annotation (13, 14). In addition, both clinical trials included a control group (i.e. CAP as control group for CAPIRI and 5FU as control group for FOLFIRI), which is required to distinguish predictive from prognostic markers. Furthermore, biomarker independence was tested by including potential confounding factors in the statistical models. Nonetheless, after initial validation in a second subsample of the CAIRO study, we were not able to validate the negative predictive value of DCR1 methylation for irinotecan-based therapy in the independent patient series from FOCUS. A lack of correlation between DCR1 methylation and DCR1 gene expression could be one of the reasons why we were not able validate DCR1 methylation as marker for response to irinotecan-based therapy. However, our cell line experiments as well analysis of a large series from the TCGA database did show a correlation between DCR1 DNA methylation and gene expression silencing. All this data together obviously raised the question whether DCR1 methylation should simply be discarded as potential biomarker for response to irinotecan-based therapy, or whether our findings can be explained otherwise.

The two trials for instance, while they show substantial resemblances at first glance differ in a number of features related to inclusion (e.g. the performance scores leading to differences in patient characteristics) and treatment (e.g. different backbone treatment; CAP versus 5FU). In addition, potential differences in the collection and storage of material may affect the results of analytical procedures. However, one could argue that a predictive biomarker of clinical value should be robust enough to cope with these variations. On the other hand, it is well known that standardization in sample handling and processing is critical also in the field of mRNA profiling and NGS(31-33).

The current study has some limitations as well. For example, measurements were performed on samples from the primary tumor, while patients were treated for their metastases, which provokes the question whether intra tumor heterogeneity could play a role. Although metastases can acquire additional genomic alterations, they keep most alterations present in the primary tumor (34, 35). As DNA methylation usually is early event in colorectal carcinogenesis, this is a likely to be the case here as well(36).
Another limitation of the current study is that $DCR1$ methylation analyses were performed with identical primers but with different reagents in different laboratories for the three study cohorts. This could have introduced variability in test results. The proportion of patients having a positive test result was slightly different for the three cohorts indeed (39% in the discovery set, 53% in the internal validation set and 48% in the external validation set). However, because the predictive value of $DCR1$ methylation with regard to irinotecan-based therapy showed similar results in the two cohorts with largest relative difference in prevalence of methylation (39% vs 53%), this variability is not likely to be the cause of the inability to validate $DCR1$ methylation as a predictive biomarker.

In conclusion, the present study revealed $DCR1$ methylation as a negative predictive marker for irinotecan-based therapy in metastatic colorectal cancer in both a discovery and an initial validation set, but this could not be confirmed in an external validation data set. The present study highlights the importance of extensive evaluation of potential biomarkers. It also shows the complexity and extensiveness of systematic evaluation of a potential biomarker in order to generate more than just a proof of concept, and that a well designed study is not a guarantee of success. Improvements in multi-team collaborations and in organizing data acquisition and biobanking in clinical trials will be necessary for efficient and successful discovery of predictive biomarkers in the future.
REFERENCES


### SUPPLEMENTARY TABLE 1. MSP primer sequences.

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<th>Lightcycler MSP primers</th>
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<td>BIK</td>
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SUPPLEMENTARY TABLE 2. Discovery set: observed methylation frequencies of candidate genes

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<tr>
<td>DCR1</td>
<td>39%</td>
</tr>
<tr>
<td>EEF1A2 (primer set 1)</td>
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</tr>
<tr>
<td>EEF1A2 (primer set 2)</td>
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</tr>
<tr>
<td>HOXA9</td>
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<tr>
<td>IRAK1</td>
<td>40%</td>
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<td>LIG4</td>
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<td>NUDT1</td>
<td>92%</td>
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SUPPLEMENTARY FIGURE 1. CAIRO discovery set: Progression-free survival in metastatic colorectal cancer patients with unmethylated (U; blue lines) or methylated (M; red lines) DCR1 (A and B) or IRAK1 (B and C) treated in first line with capecitabine, oxaliplatin and bevacizumab. HR=Hazard Ratio (M versus U).