EUROPEAN JOURNAL OF CANCER 47 (2011) 839–847

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Association of RRM1 –37A>C polymorphism with clinical outcome in colorectal cancer patients treated with gemcitabine-based chemotherapy

J. Rodriguez [a,c], V. Boni [a,b,c], A. Hernández [a], N. Bitarte [b], R. Zarate [b], M. Ponz-Sarvisé [a], A. Chopitea [a], E. Bandres [b,*], J. Garcia-Foncillas [a,b,*]

[a] Department of Oncology, Clinic University of Navarra, Pamplona, Spain
[b] Laboratory of Pharmacogenomics, Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain

ARTICLE INFO

Article history:
Received 10 September 2010
Received in revised form 26 November 2010
Accepted 30 November 2010
Available online 8 January 2011

Keywords:
Gemcitabine
Metastatic colorectal cancer
RRM1
CDA
Polymorphism

ABSTRACT

Background: To investigate whether single nucleotide polymorphisms (SNPs) in gemcitabine (GMB) metabolism genes were associated with clinical outcome in pre-treated metastatic colorectal cancer (mCRC) patients.

Patients and methods: SNPs of hCNT1, hENT1, CDA, dCTD and RRM1 genes were evaluated in 95 mCRC patients and detected using TaqMan genotyping assays. Association of genotypes with overall response rate (ORR), time to progression (TTP) and overall survival (OS) was tested by univariate and multivariate analysis. RRM1 –37A>C polymorphism was correlated with GMB IC50 value and with the RRM1 gene expression level in CRC cell lines.

Results: The ORR was 38.9%. The median TTP and OS were 4 and 14.3 months, respectively. By multivariate analysis, patients carrying the RRM1 –37CC genotype or the CDA A-76 C-containing allele had a significantly higher likelihood of achieving a tumour response. RRM1 –37A>C polymorphism remained associated with clinical efficacy (TTP). *In vitro* experiments, in CRC cell lines, showed that the RRM1 A-37C genotype was associated with the levels of RRM1 expression and with GMB IC50 values. Finally, the down-regulation of RRM1 with a specific siRNA strongly influenced GMB sensitivity.

Conclusion: RRM1 –37A>C polymorphism may represent a useful biomarker to select mCRC patients most likely to benefit from GMB-based salvage therapy.

1. Introduction

Over the last years, an improvement in the outcome of metastatic colorectal cancer (mCRC) patients has been achieved with the use of polychemotherapy regimens combining 5-fluorouracil (5-FU) with either irinotecan or oxaliplatin, alone or in combination with monoclonal antibodies.[1] However, resistance of cancer cells to standard therapeutic options remains a major clinical obstacle, and the continuing search for alternative active combinations is therefore justified.

Several phase I/II trials of single agent gemcitabine (GMB) have demonstrated only a modest activity in mCRC patients.[2,3] However, clinical outcomes remarkably improve when GMB is used in combination regimens.[4–6] A growing

* *Corresponding authors:* Address: Laboratory of Pharmacogenomics, Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Avda Pio XII 55, 31008-Pamplona, Spain. Tel.: +34 948194700.
E-mail addresses: ebandres@unav.es (E. Bandres), jgfoncillas@unav.es (J. Garcia-Foncillas).
[c] Javier Rodríguez and Valentina Boni have equally contributed.
0959-8049/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2010.11.032

body of *in vitro* evidence suggests that GMB synergistically interacts with some of the most active agents in mCRC, including 5-FU, probably due to the GMB-enhancement of 5-FU uptake in target cells, and oxaliplatin, through a GMB-induced blockade of the nuclear mechanisms used by cancer cells to repair platinum-related intra-strands cross links.[7] GMB also increases the antitumour activity of oxaliplatin and 5-FU due to the activation of caspase 3 and 9-dependent apoptosis and through a decreased expression of Raf-1 and AKT, enhancing the process of programmed cell death individually activated by each agent.[8] A synergistic sequence-dependent interaction of GMB and SN-38 has also been proposed in preclinical models, owing to the capacity of the combination to overcome S-phase checkpoint-mediated resistance.[9] The incorporation of GMB into DNA enhances campothecin-induced topoisomerase-1 cleavage complexes, leading to an improved cytotoxic activity.[10] Finally, blockade of VEGF-receptor activation has been shown to enhance the efficacy of GMB in preclinical models,[11] and a recent work demonstrated that GMB increases the epidermal growth factor-receptor (EGFR) expression on the surface of CRC cell lines and, consequently, the cetuximab mediated antibody-dependent cell cytotoxicity.[12]

This preclinical background has prompted the design of several trials with GMB-based combinations in pre-treated mCRC patients (Table 1), showing an interesting tumour growth control rate and a favourable toxicity profile.[13]

GMB is a nucleoside analogue and a prodrug that requires cellular uptake and intracellular phosphorylation and is transported into the cells mostly by human equilibrative nucleotide transporter-1 and 2 (hENT) and human concentrative nucleotide transporters 1–3 (hCNT). GMB is metabolised intracellularly to gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP). Deoxycytidine kinase (dCK) is the rate-limiting enzyme of this biotransformation. GMB exerts its cytotoxic effects through incorporation of dFdCTP into DNA, with the subsequent DNA synthesis inhibition and the induction of apoptosis. In addition, dFdCDP interferes with ribonucleotide reductase (RR), causing a depletion of deoxynucleotide triphosphates necessary for DNA synthesis. GMB is inactivated primarily by deoxycytidine deaminase (CDA) into difluorodeoxyuridine, and GMB monophosphate is inactivated by deoxycytidylate deaminase (dCTD).[14]

Previous *in vitro* works have associated GMB resistance to decreased expression of the activation enzyme, increased degradation, decreased nucleoside transport of drug into cells and increased expression of the ribonucleotide reductase subunit 1 (RRM1).[15] Clinical studies have shown associations of polymorphic variants of GMB metabolic, transporters and drug's target genes with toxicity and clinical outcome in solid tumours, mainly NSCLC[16] and pancreatic cancer.[17]

In the current study, we retrospectively evaluated the correlation of hCNT1, hENT1, CDA, DCTD and RRM1 polymorphisms with clinical outcome in 95 pre-treated mCRC patients. *In vitro* work was carried out to confirm our clinical findings.

2. Patients and methods

2.1. Patients' recruitment and data collection

Patients with histologically proven mCRC in failure after at least one previous systemic therapy for metastatic disease and with a DNA sample available were included. Additional eligibility criteria included age > 18 years, an Eastern Cooperative Oncology Group (ECOG) performance status score of 0–2, a life expectancy > 12 weeks and an adequate organ function (absolute neutrophil count > 1.5×10^9/l, a platelet count > 75×10^9/l, serum creatinine < 1.3 mg/dl, serum bilirubin < 1.25 times the normal upper limit (UNL) and serum transaminases < 3.0 times UNL). The main exclusion criteria were active second malignancy (except for non-melanoma skin cancer or in situ cervical cancer), symptomatic metastases in the central nervous system or carcinomatous leptomeningitis, uncontrolled severe infection or major organic failure.

2.2. Treatment

The chemotherapy regimen consisted of GMB, 1000 mg/m^2 administered at an infusional fixed dose-rate of 10 mg/m^2/min on a biweekly basis, followed by oxaliplatin (21 patients), irinotecan (58 patients) or fluoropyrimidines (16 patients); the addition of a targeted therapy was allowed according to the physician criteria, and included cetuximab (24 patients) and bevacizumab (35 patients).The drugs combined with GMB were chosen based on the previous lines of therapy received by each patient, and the study regimens were administered through a regulated compassionate-use programme.

The local institutional review board approved the study and all patients provided written informed consent before recruitment.

Table 1 – Previous clinical studies with gemcitabine in pre-treated mCRC.

Regimen (study reference)	*n*	Setting	ORR	TTP	OS
GOLF[6] (GMB-FOLFOX)	29	2nd line	41.5%	7.2	22
GOLFIG[1] (GOLF + GM-CSF + IL-2sc)	46	2nd–3rd line (12 patients chemonaive)	56.5%	12.2	18.6
GEMOX[30]	34	2nd line (CPT-11 pre-treated)	17.7% (DCR; 41.2%)	2.7	9.1
GMB-fluoropyrimidines (pooled analysis)[13]	216	1st–3rd line	30–38.3%	4–8.3	9.8–18
Gemctabine-5-FU CI[5]	37	3rd line Oxaliplatin and CPT-11 pre-treated	10.8% (DCR; 62.2%)	4.2	8.9
GEMOX (FDR-GMB)[31]	10	2nd-line (CPT-11 pre-treated)	20%	3.7	NS

CI: continuous infusion. FDR: fixed dose rate. DCR: disease control rate. ORR: overall response rate. TTP: time to progression. OS: overall survival. CPT-11: irinotecan. NS: not supplied.

2.3. Evaluation criteria

Pre-treatment baseline evaluation included a complete medical history, physical examination, full blood count, biochemistry including carcinoembryonic antigen (CEA) and a computed tomography (CT) of chest, abdomen and pelvis. Physical examination and blood cell counts were performed biweekly. Tumour response was evaluated by CT every 8 weeks and defined according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria.[18] All responses had to be confirmed 28 d or more after the initial documentation of response. At the time of maximum response, patients were evaluated by a multidisciplinary team to rule out whether a consolidative approach should be attempted. These approaches consisted of surgical removal of all macroscopic remaining disease, radiofrequency ablation or liver radioembolisation with Yttrium^{90} microspheres.

2.4. DNA extraction and genotyping

Five polymorphisms in genes associated to the metabolism and mechanism of action of gemcitabine (Table 2) were selected on the basis of the following criteria: (1) the SNPs should reside in a functional region including exons, promoters or UTRs regions, (2) the SNPs should have reported minor allelic frequency greater than 0.20 in Caucasian population as recorded in SNP database (http://www.ncbi.nlm.nih.gov/SNP).

DNA was extracted from EDTA-anticoagulated peripheral blood using the DNAeasy Mini kit (Qiagen). Candidate SNPs were genotyped with Taqman-based real-time polymerase chain reaction (PCR) using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Primers and probes were obtained from Applied Biosystems as Assays-on-Demand SNP genotyping product (Table 2).

2.5. Cell culture and reagents

The human CRC cell lines RKO, LoVo, DLD1, HCT15, LS174, Caco2, HT29, T84, LS411'N, WiDr, SW1417, Colo201, SW948, HCT116, SW480, SW1116, LS513 and SW620 were obtained from the American Type Culture Collection (ATCC). All cell lines were maintained in the recommended medium supplemented with 1% penicillin–streptomycin and 10% FBS (GIBCO-BRL).

2.6. qRT-PCR analysis of mRNA expression

Total RNA was isolated from cell lines by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. In siRRM1-transfected cells, total RNA was extracted at 48 h after transfection. cDNA was synthesised with the High-Capacity cDNA Synthesis kit (Applied Biosystems) using 2 μg total RNA as the template and random primers. The PCR primers and probes for RRM1 (Hs01040705_m1) and the internal control gene RNAr 18s (Hs 99999901_s1) were purchased from Applied Biosystems.

2.7. Transfections of small interfering RNA specific to RRM1

Small interfering RNA (siRNA) specific to RRM1 (On-Target plus SMART pool L-004270-00-0005) and control siRNA (ON-TARGETplus Non-targeting pool D-001810-10-05) were designed and synthesised by Dharmacon. Transfection was performed with Lipofectamine 2000 (Invitrogen). Specific si-RNA for RRM1 and the control siRNA were transfected into DLD-1 and HCT-15 colon cancer cell lines grown in six-well dishes (plated at 2.5×10^5 cells per well 24 h before transfection). Transfection efficiency (>95%) was confirmed using the Silencer FAM-labelled Negative Control (Ambion).

2.8. Western blot

At 48 h after transfection with siRRM1 and control siRNA, protein was harvested. Lysates (50 μg) were separated using SDS–PAGE (Invitrogen). Immunoblotting of antibodies specific for RRM1 (RRM1, rabbit monoclonal antibody, Cell Signaling) and β-tubulin (Tub 2.1, mouse IgG_1, Sigma) were detected using HRP-conjugate anti-rabbit and HRP-conjugated anti-mouse antibody (Sigma), respectively. Detection was done using the chemoluminescence detection systems (PerkinElmer LAS).

2.9. Gemcitabine treatment in vitro and cytotoxicity assay

Twenty-four hours after transfection with a non-specific control siRNA, or siRRM1, all cell lines were plated in 96-well plates at 5×10^3 cells per well in triplicate. After 24 h, an additional 100 μl of medium containing gemcitabine was added, and the cells were cultured for an additional 72 h. The concentration of gemcitabine varied from 5×10^{-9} M to 10×10^{-6} M. After 72 h, cell viability was measured with a CellTiter 96 Aqueous assay kit (Promega) according to the manufacturer's instructions. The results were expressed as a percentage of cell survival on the basis of the difference with the OD in the control (no drug exposure). IC50, the drug concentration that inhibits 50% of the cell growth, was calculated by SPSS (San Diego, CA).

Table 2 – Polymorphisms in gemcitabine metabolism genes selected in this study.

Gene	SNP	Applied Biosystem Assay ID	dbSNP ID	Minor allele frequency[a]
SLC28A1 (hCNT1)	Ex9-9C>A, Q237K	C__25971678_30	rs8187758	0.20
SLC29A1 (hENT1)	IVS2+913C>T	C___2735230_10	rs9394992	0.32
CDA	Ex2-76A>C, K27Q	C__25472931_20	rs2072671	0.44
DCTD	Ex4-47T>C, V116V	C_1842730_20	rs7663494	0.33
RRM1	A-37C	C_2769831_10	rs12806698	0.27

[a] Allele frequencies (Caucasian) were from the SNP database.

2.10. Statistical analysis

Patient characteristics were reported as frequencies and percentages or as medians and interquartile range as appropriate. Groups were compared using the Pearson chi-square test for categorical variables and *U*-Mann–Whitney for continuous variables (two-tailed). The genotype distribution was tested for Hardy–Weinberg equilibrium using the goodness-of-fit χ^2 test. The significant factors associated with tumour response to treatment in the univariate analysis were analysed by logistic regression models in the multivariate analysis.

TTP and OS were calculated from the first day of treatment to the date of first observation of progressive disease and death, respectively. Patients who underwent consolidation procedures were censored at that time for TTP analysis but not in the OS analysis in which consolidative procedures were considered as a confounder factor. Kaplan–Meier analysis of survival was used to calculate median TTP and OS, and the log-rank or Breslow tests were applied to test the differences in time-to-event across different genotypes. The significant prognostic variables in the univariate analysis were included in the multivariate analyses using Cox's proportional hazards model to identify factors of independent significance. We estimated the false-positive report probability (FPRP) for the observed statistically significant associations using the methods described by Wacholder et al.[19] In the current study, we set the odds ratio (OR) and HR values of 2.0–4.0 as a likely threshold value. The prior probability used was 0.25 for all SNPs. The FPRP value for noteworthiness was set at 0.2, which indicates that any finding with an FPRP *P* value of <0.2 is noteworthy.

All statistical tests were performed with the SPSS software v15.0 for Windows (SPSS Inc., Chicago). *P*-values lower than 0.05 were considered statistically significant.

3. Results

3.1. Patient characteristics and clinical predictors of outcome

Characteristics of the 95 Caucasian patients included in the analysis are described in Table 3. All patients were in failure after at least one prior chemotherapy regimen for metastatic disease and 35% of the patients received the GMB-based regimen as third or further line of therapy. All patients received prior therapy fluoropyrimidines, whereas 98%, 55% and 30% had received oxaliplatin, irinotecan and cetuximab, respectively. Patients were considered refractory to the previous line of therapy if PD was documented as the best response. Patients who achieved an objective response (CR/PR) or SD but progressed during or within 3 months thereafter from the end to the previous therapy were considered to have a resistant disease. In 40% of cases, the disease was considered refractory or resistant to the preceding line of therapy.

On an intent-to-treat basis, overall response rate was 39%, with 35 partial and 2 complete responses, respectively. After a median follow-up of 40 months (range: 14.3–63.9), the median TTP and OS were 4 (95% CI: 0.3–32.8) and 14.3 months (95% CI: 0.3–63.9), respectively.

Table 3 – Baseline patients' characteristics.

Characteristic	Number of patients (%)
Patients	95
Median age (years; range)	59 (35–77)
Gender	
Male	59 (62)
Female	36 (38)
ECOG performance status	
0	15 (16)
1	63 (66)
2	17 (18)
Primary tumour site	
Colon	64 (67)
Rectum	31 (33)
Number of metastatic sites (median; range)	2 (1–6)
Number of prior CT for metastatic disease (median; range)	1 (1–3)
Köhne risk classification	
Low risk	30 (32)
Intermediate risk	45 (47)
High risk	20 (21)
Disease status	
Sensitive	57 (60)
Resistant/refractory	38 (40)
Most commonly used prior regimens	
FOLFOX-Cetuximab	28 (29)
FOLFIRINOX	43 (45)
XELOX/FOLFOX	26 (27)
FOLFIRI-Bevacizumab	13 (14)
Mitomycin-Capecitabine	8 (8)

Both Köhne risk index[20] and response to the previous line of therapy were associated with ORR and TTP in the univariate analysis (Table 4).

3.2. Correlation between polymorphisms, response to chemotherapy and disease control rate

All polymorphisms followed the Hardy–Weinberg's equilibrium and genotype frequencies were comparable with those reported in previous studies.

No significant correlations were observed between hCNT1, hENT1 or DCTD genotypes and ORR (Table 4). Individually, a significant correlation between tumour response and the polymorphisms analysed was found for CDA A-76C and RRM1 A-37C genotypes. In particular, in patients carrying a CDA C-containing allele the ORR was 48%, compared to only 24% in carriers of the homozygous CDA A variant ($P = 0.02$). Similarly, 52% of the patients that experienced response to therapy harbour the RRM1 –37 C/C genotype, whereas only 18% of them carried an RRM1 –37 A-containing allele ($p = 0.001$). Moreover, when CDA A-76C and RRM1 A-37C were analysed in combination, a gene-dosage effect was observed. Overall response rate for patients carrying 2, 1 or 0 favourable polymorphisms was 61%, 35% and 7%, respectively ($P = 0.004$).

Table 4 – ORR and TTP according to clinical factors and genotypes.

	Responders (CR + PR)	No Responders (SD + P)	P[a]	*n* (%)	TTP (median)	P[b]
Age						
<60	22 (43%)	29 (57%)	0.367	51 (54%)	5.4 (3.8–6.9)	0.490
⩾60	15 (34%)	29 (66%)		44 (46%)	4.8 (4.1–5.6)	
Sex						
Male	22 (37%)	37 (63%)	0.671	59 (62%)	5.4 (4.2–6.5)	0.692
Female	15 (42%)	21 (58%)		36 (38%)	4.9 (4.3–5.5)	
Primary tumour site						
Colon	25 (39%)	39 (61%)	0.974	64	4.8 (4.2–5.5)	0.092
Rectum	12 (39%)	19 (61%)		31	5.8 (3.7–7.9)	
Prior QT treatment						
1 lines	26 (42%)	36 (58%)	0.413	62 (65%)	5.1 (4.3–6.0)	0.903
⩾2 lines	11 (33%)	22 (67%)		33 (35%)	4.6 (3.9–5.3)	
Köhne index						
Low	20 (67%)	10 (33%)	**<0.001**	30 (32%)	7.5 (6–8.9)	**0.006**
Intermediate	15 (34%)	29 (66%)		45 (47%)	5.0 (3.9–6.0)	
High	2 (10%)	19 (90%)		20 (21%)	1.6 (1.6–2.4)	
Response to previous therapy						
Sensitive	28 (49%)	29 (51%)	**0.013**	57 (60%)	6.4 (5.0–7.9)	**<0.001**
Resistant and refractory	9 (24%)	29 (76%)		38 (40%)	2.8 (0.9–4.7)	
hCNT1 C-9A(Q237K)						
C/C	21 (40%)	31 (60%)	0.752	52	5 (4.3–5.7)	0.840
C/A+A/A	16 (37%)	27 (63%)		43	5.2 (4.2–6.1)	
hENT1 C+913T						
C/C	22 (41%)	32 (59%)	0.903	54	5.1 (3.9–6.3)	0.652
C/T+T/T	15 (39%)	23 (61%)		38	5.0 (3.7–6.3)	
CDA A-76C (K27Q)						
A/A	9 (24%)	28 (76%)	**0.020**	38 (40%)	4.4 (3.6–5.3)	**0.024**
A/C+C/C	28 (48%)	30 (52%)		57 (60%)	5.6 (3.7–7.5)	
DCTD T-47C (V116V)						
T/T	21 (40%)	34 (60%)	0.858	55	5.0 (4.3–5.7)	0.731
T/C+C/C	16 (40%)	24 (60%)		40	5.1 (3.9–6.2)	
RRM1 A-37C						
A/A+A/C	6 (18%)	28 (82%)	**0.001**	34 (36%)	4.1 (1.8–6.5)	**0.021**
C/C	31 (52%)	29 (48%)		59 (63%)	5.4 (3.4–7.3)	
Combined favourable CDA-76 and RRM1 –37						
None favourable	1 (7%)	13 (93%)	**0.004**	14 (15%)	1.8 (0.1–4.0)	**0.005**
1 favourable	16 (35%)	30 (65%)		43 (49.5%)	5.1 (3.3–6.9)	
2 favourable	29 (61%)	14 (39%)		38 (35.5%)	6.6 (3.9–9.3)	

[a] Difference of the estimates tested using Pearson chi-square. Bold characters indicate statistically significant results.
[b] Difference of Kaplan–Meier estimates of TTP tested using the log-rank or Breslow test. Bold characters indicate statistically significant results.

By logistic regression, Köhne risk index, CDA A-76C and RRM1 A-37C genotypes were all independently associated with ORR (Table 5). In the multivariate analysis, the presence of both CDA and RRM1 favourable genotypes was significantly associated to ORR ($P = 0.005$). The FPRP was 0.163 for patients carrying two favourable genotypes, indicating noteworthiness.

3.3. *Correlation between polymorphisms and clinical outcome*

As shown in Table 4, patients harbouring either a CDA-76 C-containing genotype or the RRM1 –37C/C genotype had a longer median TTP than those with alternative genotypes (Fig. 1a and b). When CDA A-76C and RRM1 A-37C were analysed in combination, TTP increased as the number of favourable alleles increased. Median time to progression in patients carrying 2, 1 or none of the favourable polymorphisms (CDA-76C-containing genotype and RRM1 –37C/C) was 6.6 months (95% CI: 3.9–9.3), 5.1 months (95% CI: 3.3–6.9) and 1.8 months (95% CI: 0.1–4.0), respectively ($P = 0.005$).

As shown in Table 6, after adjusting for the significant clinical factors, RRM1 A-37C genotype remained as an independent predictor of TTP. Patients harbouring an RRM1 37 A-containing genotype had a 1.9-fold risk of progression compared to those carrying the homozygous C variant. The FPRP

Table 5 – Multivariate analysis for polymorphisms and clinical factors associated with ORR.

Factor	Variable	ORR OR (95% CI)	P
Köhne index	High Low + Intermediate	1 8.9 (1.7–44.7)	**0.008**
CDA A-76C	A/A A/C + C/C	1 3.2 (1.1–8.9)	**0.027**
RRM1 A-37C	A/A + A/C C/C	1 5.7 (1.8–16.7)	**0.002**
Combined favourable CDA and RRM1 genotypes	0–1 2	1 4.0 (1.5–10.6)	**0.005**

Odds ratio (OR) with the 95% confidence interval (95% CI) and the p value were estimated using the logistic regression model. Bold characters indicate statistically significant results.

Table 6 – Multivariate analysis for the polymorphism A-37C in RRM1 gene and clinical factors associated with TTP.

Factor	Variable	TTP HR (95% CI)	P
Köhne index	Low + intermediate High	1 1.9 (1.1–3.5)	**0.026**
Response to previous therapy	Sensitive Resistant and refractory	1 1.9 (1.1–3.4)	**0.014**
OR	CR + PR SD + P	1 7.6 (3.9–14.8)	**<0.001**
RRM1 A-37C	C/C A/A + A/C	1 1.9 (1.1–3.4)	**0.021**

Hazard ratio (HR) with the 95% confidence interval (95% CI) and the P value were estimated using the Cox regression model. Bold characters indicate statistically significant results.

for patients carrying the RRM1 –37C/C genotype was 0.139, indicating noteworthiness.

The CDA –76C and RRM1 –37C genotypes also correlated with a statistically significant longer median OS. However, multivariate analysis failed to demonstrate this association (data not shown).

None of the other SNPs showed significant associations with TTP (Table 4).

3.4. *RRM1 promoter polymorphisms and RRM1 expression in CRC cell lines*

To determine whether the RRM1 A-37C genotype was associated with the levels of RRM1 expression in CRC cell lines, RRM1-mRNA was quantified by qRT-PCR in 18 CRC cell lines. Normalised RRM1 gene expression was associated to RRM1 gene promoter variant genotype (A-37C). The lowest RRM1 expression was detected in colon cell lines harbouring the RRM1 –37C/C genotype ($P = 0.072$) (Fig. 2a). To gain insight into the functional role of RRM1 A-37C polymorphism in GMB sensitivity, the GMB IC50 was calculated in 4 colon cancer cell lines. GMB IC50 in those cell lines carrying the RRM1 –37 A-containing genotype (HCT15 and DLD1) was almost 2-fold higher than in cell lines carrying the RRM1 –37 C/C genotype (HCT116 and LoVo). In particular, HCT15 and DLD1 CRC cell lines had IC50 values of 21.6 ± 2.7 nM and 74 ± 12.5 nM, respectively, compared to 11.9 ± 1.1 nM and 6.8 ± 1.5 nM, respectively, for HCT116 and LoVo colon cancer cell lines. Finally, to rule out whether RRM1 was a determinant of GMB resistance *in vitro*, a small interfering RNA specific to RRM1 was transfected in HCT15 and DLD1 cell lines. This RRM1-siRNA efficiently decreased both RRM1 mRNA and protein levels (Fig. 2b and cc). We found that the siRNA-induced RRM1 down-regulation resulted in a higher GMB sensitivity as compared to control siRNA in both HCT15 and DLD1 CRC cell lines. GMB IC50 values were in cell transfected 34.3 ± 1.2 nM versus 8.5 ± 1.5 nM for HCT15 cell line and 76.0 ± 1.48 nM versus 15.85 ± 0.8 nM in DLD1 cell lines (Fig. 2d).

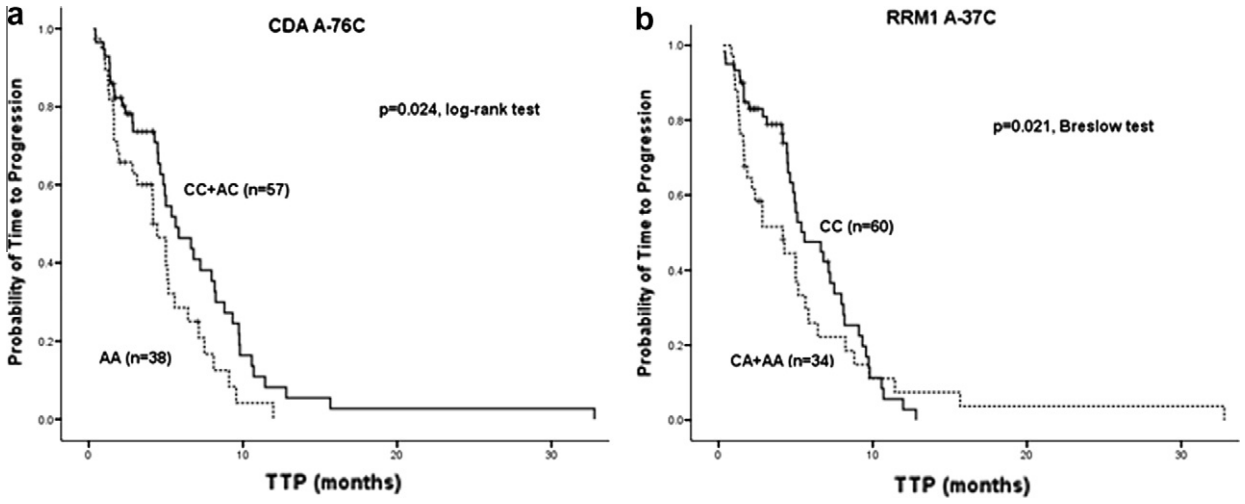

Fig. 1 – Association between SNPs in CDA and RRM1 genes on TTP in mCRC patients. Kaplan–Meier method was used to assess the effect of polymorphisms in CDA (a) and RRM1 (b) genes on time to progression (TTP) in 95 patients with mCRC treated with gemcitabine-based therapy.

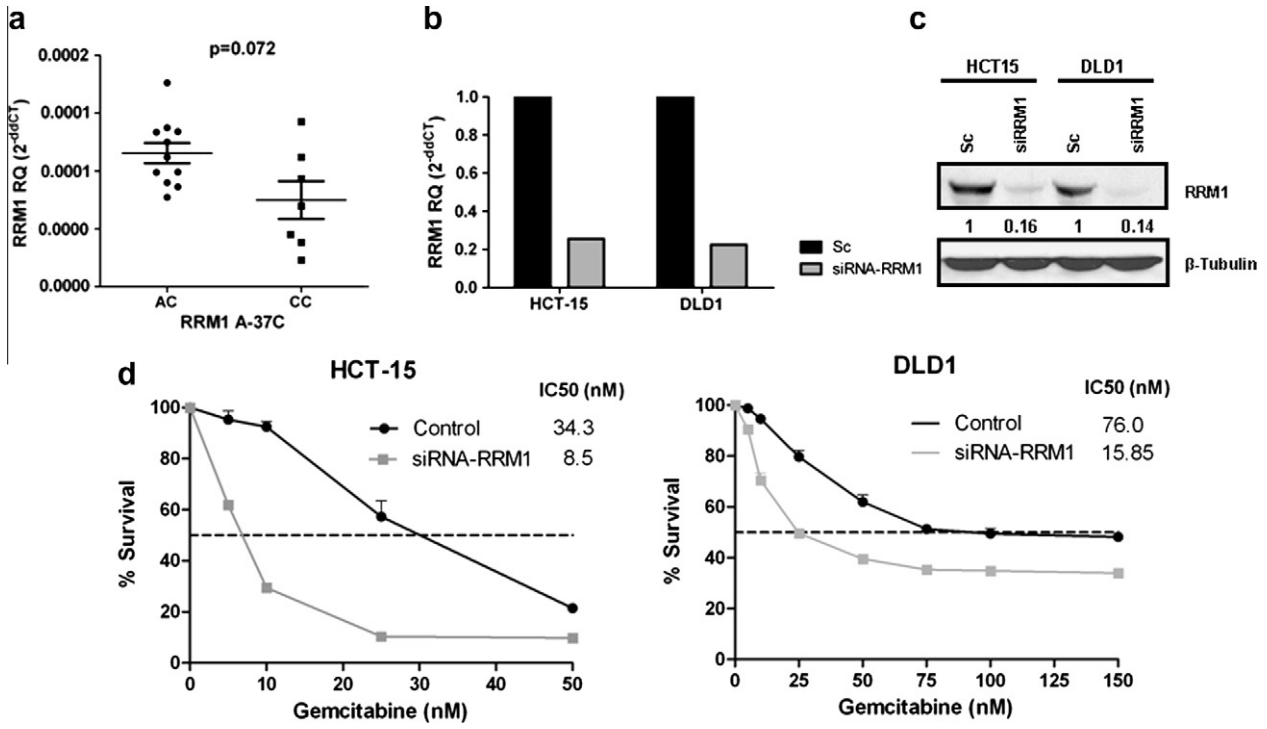

Fig. 2 – Influence of RRM1 on gemcitabine sensitivity in CRC cell lines. (a) Expression analysis of RRM1 mRNA in a panel of CRC cell lines with different RRM1 A-37C genotype. Data are expressed as $2^{-\Delta\Delta Ct}$ values obtained by normalisation using RNA18S as endogenous control. (b) Expression analysis of RRM1 in HCT15 and DLD1 cells transfected with a non-specific siRNA and with siRNA-RRM1. Data are expressed as $2^{-\Delta\Delta Ct}$ values obtained by normalisation using RNA18S as endogenous control and cells transfected with a non-specific siRNA control as calibrator. (c) Western-blot of RRM1 protein expression in HCT15 and DLD1 cells transfected with a non-specific siRNA control or siRNA-RRM1; β-tubulin was used as protein loading control. (d) Chemosensitivity to GMB of HCT15 and DLD1 CRC cells transfected with a non-specific siRNA or siRNA-RRM1 was measured using a MTS assay kit. The results were expressed as the percentage of cell survival on the basis of the difference between the OD of untreated cells and treated cells. Each point represents the mean of three different experiments.

4. Discussion

To date, several authors have addressed the role of gemcitabine-based chemotherapy in patients with relapsed mCRC, with results overlapping those of the present study. Although in this setting most patients receive second or further lines of therapy, only a subset of them seem to benefit from this approach, and thus the development of polymorphism assays using germline DNA seems appealing for customising chemotherapy. To our knowledge, the results of this study are the first to support the hypothesis that genetic variations in genes associated with GMB metabolism and mechanism of action might identify pre-treated mCRC patients more likely to benefit from a GMB-based salvage therapy. We found that both the CDA A-76C and the RRM1 A-37C polymorphisms were associated to ORR, and so it was RRM1 A-37C polymorphism with TTP. Importantly, our *in vitro* results showed a different GMB chemosensitivity profile when a siRNA specific to RRM1 was transfected in the CRC cell lines analysed.

The RRM1 gene encodes the regulatory subunit of ribonucleotide reductase, an enzyme whose increased expression has been correlated with gemcitabine resistance.[20–22] *In vitro*, the SNPs A-37C and C-524T, located in the promoter region of the RRM1 gene, affected its transcriptional activity, with higher levels seen in patients with the variant allele, but no association was found between these allelotypes and RRM1 tumour expression.[23] In the clinical setting, some reports have correlated the RRM1 –37 AC genotype with a longer PFS, but not with radiological response,[16] whereas others have reported no association[24] or a worse outcome for patients harbouring the RRM1 –37 A-containing variants.[25] Our results showed that the RRM1 –37 CC genotype predicted a higher ORR and a longer median TTP. Moreover, RRM1 –37 CC remained an independent predictive variable of treatment efficacy. The hazard ratio of progression was 1.9 for patients carrying the RRM1 A-containing genotype, whereas patients with the RRM1-CC genotype had a 5.7-fold higher probability of responding to therapy.

To further validate our findings, we calculated the GMB IC50 in CRC cell lines according to the different RRM1 A-37C variant genotypes. GMB IC50 of colon cancer cells harbouring the RRM1 –37 CC genotype was lower than that of cells with the RRM1 A-containing genotype, indicating a higher GMB sensitivity in CRC cell lines with the CC variant. Moreover, we found a trend towards an association between RRM1 mRNA expression and the RRM1 A-37C polymorphism, with the lowest expression in the homozygous C variant. Finally, to confirm the role of RRM1 expression as a determinant of GMB resistance, CRC cell lines were transfected with a specific si-RRM1. Interestingly, downregulation of RRM1 expression

remarkably increased GMB sensitivity, supporting our clinical findings.

A gene–dosage effect on radiological response was observed when RRM1 A-37C and CDA A-76C genotypes were jointly analysed. Cytidine deaminase plays a key role in the metabolic inactivation of GMB.[26] Several functional SNPs in the promoter and coding region of the human CDA gene have been identified but their role is still controversial.[17] *In vitro* data suggested that the common CDA 79 A > C (Lys 27 Gln) polymorphism resulted in a moderate decrease in the level of CDA activity for the polymorphic variant and a subsequent decrease in the deamination of GMB.[27] In agreement with this functional study, we found that the CDA A-76C variant C allele was associated with a higher response rate. Alternatively, in the study by Okazaki et al. CDA-76C variant C allele was associated with a better OS, suggesting that the reduced enzyme activity conferred by this allele turned into a higher level of dug availability.[17] On the contrary, Tibaldi et al.[28] found that, among Caucasian NSCLC patients treated with cisplatin and GMB, CDA 27 Lys/Lys genotype predicted for a better clinical outcome than alternative genotypes. Importantly, these authors performed an analysis of CDA enzymatic activity in blood samples, showing that CDA activity was enhanced by polymorphic variants. Hence, CDA might be a key enzyme in the mechanism of inactivation of GMB, but the functional role of its polymorphism warrants further investigations.

Patients in the present study were classified according to the Köhnés prognostic groups.[29] This index was firstly applied as a prognostic indicator for mCRC patients treated with a front-line 5-FU-based therapy. Our findings suggest that this model might also be implemented in pre-treated mCRC patients as a simple and reliable prognostic tool. Moreover, Köhne and colleagues suggested that molecular biomarkers should be validated against these clinical parameters. In our analysis, the RRM1 A-37C polymorphism remained significantly predictive of patients' outcome even when adjusted for this index.

In summary, to our knowledge this is the first *in vivo* study to analyse the predictive role of several SNPs that may affect key genes involved in GMB mechanisms of action in a subset of mCRC patients. Our results suggest that clinical and molecular parameters may be combined to more precisely identify a subset of patients who might benefit from a GMB-based therapy.v

Conflict of interest statement

None declared.

REFERENCES

1. Correale P, Cusi MG, Tsang KY, et al. Chemo-immunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. *J Clin Oncol* 2005;**23**(35):8950–8.
2. Moore Jr DF, Pazdur R, Daugherty K, Tarassoff P, Abbruzzese JL. Phase II study of gemcitabine in advanced colorectal adenocarcinoma. *Invest New Drugs* 1992;**10**(4):323–5.
3. Mani S, Kugler JW, Knost JA, et al. Phase II trial of 150-minute weekly infusion of gemcitabine in advanced colorectal cancer: minimal activity in colorectal cancer. *Invest New Drugs* 1998;**16**(3):275–8.
4. Madajewicz S, Hentschel P, Burns P, et al. Phase I chemotherapy study of biochemical modulation of folinic acid and fluorouracil by gemcitabine in patients with solid tumor malignancies. *J Clin Oncol* 2000;**18**(20):3553–7.
5. Bitossi R, Sculli CM, Tampellini M, et al. Gemcitabine and protracted 5-fluorouracil infusion as third-line chemotherapy in refractory colorectal cancer patients. *Anticancer Res* 2008;**28**(5B):3055–60.
6. Correale P, Messinese S, Caraglia M, et al. A novel biweekly multidrug regimen of gemcitabine, oxaliplatin, 5-fluorouracil (5-FU), and folinic acid (FA) in pretreated patients with advanced colorectal carcinoma. *Br J Cancer* 2004;**90**(9):1710–4.
7. Bergman AM, Ruiz van Haperen VW, Veerman G, Kuiper CM, Peters GJ. Synergistic interaction between cisplatin and gemcitabine in ovarian and colon cancer cell lines. *Adv Exp Med Biol* 1994;**370**:139–43.
8. Caraglia M, Marra M, Budillon A, et al. Chemotherapy regimen GOLF induces apoptosis in colon cancer cells through multi-chaperone complex inactivation and increased Raf-1 ubiquitin-dependent degradation. *Cancer Biol Ther* 2005;**4**(10):1159–67.
9. Galvez-Peralta M, Dai NT, Loegering DA, et al. Overcoming S-phase checkpoint-mediated resistance. sequence-dependent synergy of gemcitabine and 7-ethyl-10-hydroxycamptothecin (SN-38) in human carcinoma cell lines. *Mol Pharmacol* 2008;**74**(3):724–35.
10. Pourquier P, Gioffre C, Kohlhagen G, et al. Gemcitabine (2′,2′-difluoro-2′-deoxycytidine), an antimetabolite that poisons topoisomerase I. *Clin Cancer Res* 2002;**8**(8):2499–504.
11. Bruns CJ, Shrader M, Harbison MT, et al. Effect of the vascular endothelial growth factor receptor-2 antibody DC101 plus gemcitabine on growth, metastasis and angiogenesis of human pancreatic cancer growing orthotopically in nude mice. *Int J Cancer* 2002;**102**(2):101–8.
12. Correale P, Marra M, Remondo C, et al. Cytotoxic drugs up-regulate epidermal growth factor receptor (EGFR) expression in colon cancer cells and enhance their susceptibility to EGFR-targeted antibody-dependent cell-mediated-cytotoxicity (ADCC). *Eur J Cancer* 2010;**46**(9):1703–11.
13. Merl M, Hoimes C, Pham T, Saif MW. Is there a palliative benefit of gemcitabine plus fluoropyrimidines in patients with refractory colorectal cancer? A review of the literature previously presented: poster at the 2008 Gastrointestinal Cancer Symposium (Abstract No. 512). *Expert Opin Investig Drugs* 2009;**18**(9):1257–64.
14. Danesi R, Altavilla G, Giovannetti E, Rosell R. Pharmacogenomics of gemcitabine in non-small-cell lung cancer and other solid tumors. *Pharmacogenomics* 2009;**10**(1):69–80.
15. Okazaki T, Jiao L, Chang P, et al. Single-nucleotide polymorphisms of DNA damage response genes are associated with overall survival in patients with pancreatic cancer. *Clin Cancer Res* 2008;**14**(7):2042–8.
16. Dong S, Guo AL, Chen ZH, et al. RRM1 single nucleotide polymorphism –37C→A correlates with progression-free survival in NSCLC patients after gemcitabine-based chemotherapy. *J Hematol Oncol* 2010;**3**(10).
17. Okazaki T, Javle M, Tanaka M, Abbruzzese JL, Li D. Single nucleotide polymorphisms of gemcitabine metabolic genes and pancreatic cancer survival and drug toxicity. *Clin Cancer Res* 2010;**16**(1):320–9.

18. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;**92**(3):205–16.
19. Wacholder S, Rothman N, Caporaso N. Population stratification in epidemiologic studies of common genetic variants and cancer: quantification of bias. *J Natl Cancer Inst* 2000;**92**(14):1151–8.
20. Bepler G, Kusmartseva I, Sharma S, et al. RRM1 modulated in vitro and in vivo efficacy of gemcitabine and platinum in non-small-cell lung cancer. *J Clin Oncol* 2006;**24**(29):4731–7.
21. Bergman AM, Eijk PP, Ruiz van Haperen VW, et al. In vivo induction of resistance to gemcitabine results in increased expression of ribonucleotide reductase subunit M1 as the major determinant. *Cancer Res* 2005;**65**(20):9510–6.
22. Rosell R, Danenberg KD, Alberola V, et al. Ribonucleotide reductase messenger RNA expression and survival in gemcitabine/cisplatin-treated advanced non-small cell lung cancer patients. *Clin Cancer Res* 2004;**10**(4):1318–25.
23. Bepler G, Zheng Z, Gautam A, et al. Ribonucleotide reductase M1 gene promoter activity, polymorphisms, population frequencies, and clinical relevance. *Lung Cancer* 2005;**47**(2):183–92.
24. Feng JF, Wu JZ, Hu SN, et al. Polymorphisms of the ribonucleotide reductase M1 gene and sensitivity to platin-based chemotherapy in non-small cell lung cancer. *Lung Cancer* 2009;**66**(3):344–9.
25. de las Penas R, Sanchez-Ronco M, Alberola V, et al. Polymorphisms in DNA repair genes modulate survival in cisplatin/gemcitabine-treated non-small-cell lung cancer patients. *Ann Oncol* 2006;**17**(4):668–75.
26. Bengala C, Guarneri V, Giovannetti E, et al. Prolonged fixed dose rate infusion of gemcitabine with autologous haemopoietic support in advanced pancreatic adenocarcinoma. *Br J Cancer* 2005;**93**(1):35–40.
27. Gilbert JA, Salavaggione OE, Ji Y, et al. Gemcitabine pharmacogenomics: cytidine deaminase and deoxycytidylate deaminase gene resequencing and functional genomics. *Clin Cancer Res* 2006;**12**(6):1794–803.
28. Tibaldi C, Giovannetti E, Vasile E, et al. Correlation of CDA, ERCC1, and XPD polymorphisms with response and survival in gemcitabine/cisplatin-treated advanced non-small cell lung cancer patients. *Clin Cancer Res* 2008;**14**(6):1797–803.
29. Kohne CH, Cunningham D, Di CF, et al. Clinical determinants of survival in patients with 5-fluorouracil-based treatment for metastatic colorectal cancer: results of a multivariate analysis of 3825 patients. *Ann Oncol* 2002;**13**(2):308–17.
30. Ziras N, Potamianou A, Varthalitis I, et al. Multicenter phase II study of gemcitabine and oxaliplatin (GEMOX) as second-line chemotherapy in colorectal cancer patients pretreated with 5-fluorouracil plus irinotecan. *Oncology* 2006;**70**:106–14.
31. Lopes G, Quesada J, Ahn E, et al. Oxaliplatin and fixedrate infusional gemcitabine in the second-line treatment of patients with metastatic colon cancer: final results of a Phase II trial prematurely closed as a result of poor accrual. *Clin Colorectal Cancer* 2007;**6**(9):641–5.