Combination of germline variations associated with survival of folinic acid, fluorouracil and irinotecan-treated metastatic colorectal cancer patients

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Aim: Germline variants could modify survival of metastatic colorectal cancer patients (mCRC). **Patients & methods:** The association of 285 haplotype-tagging SNPs in 11 candidate genes and overall survival (OS) was tested in two cohorts totalizing 417 FOLFIRI-treated mCRC. Gene expression was investigated *in vitro* and in public datasets. **Results:** In the combined cohort, *CES1* rs9921399T>C was associated with prolonged OS (hazard ratio [HR] = 0.40) whereas *ABCC1* rs17501011G>A (HR = 2.08) and *UGT1* rs1113193G>A (HR = 2.12) were associated with shorter OS ($p \leq 0.005$). A combined effect of these polymorphisms was observed with HR of 1.98–2.97 ($p < 0.05$). The *ABCC1* rs17501011A variant reduced reporter-gene activity ($p < 0.05$) whereas *ABCC1* tumor expression was associated with shorter survival ($p \leq 0.013$). **Conclusion:** We identified a combination of genetic determinants that could predict mCRC survival.

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Prognosis of metastatic colorectal cancer patients (mCRC) remains poor with a 5-year survival of 14% and a majority of patients cannot be cured with surgical resection [1,2]. A significant number of new agents have been approved in this clinical setting. Systemic chemotherapy with first-line regimens containing irinotecan or oxaliplatin leads to significant improvements in survival and similar efficacy but different toxicity profiles [2–5]. Oxaliplatin is a platinum-derived DNA-damaging agent while irinotecan exerts its cytotoxic effects through inhibition of topoisomerase I in replicating cells by inducing DNA strand breaks. New agents include the monoclonal antibody bevacizumab targeting the VEGF whereas cetuximab or panitumumab inhibit the EGFR, preventing tumor development [6,7].

The discovery of predictive markers may assist clinicians in practice for the choice of therapy. Indeed, strategies based on germline DNA markers may help optimize treatment dosing, whereas markers based on tumor biology are used for the appropriate designation of subsequent lines of palliative chemotherapy [5,8]. For irinotecan-based regimens, recent discoveries in genes related to the pharmacology of the drug, namely variants found in the *UGT1*

Table 1. Characteristics of the Canadian and Italian cohorts of metastatic colorectal cancer patients.

Characteristics	Canadian cohort (N = 167); n (%)	Italian cohort (N = 250); n (%)
Gender:		
– Males	110 (66)	162 (65)
– Females	57 (34)	88 (35)
Age (years):		
– Mean $\pm$ standard deviation	61.5 $\pm$ 10.2	60.6 $\pm$ 10.3
– Range	29–86	26–75
FOLFIRI:	167 (100)	250 (100)
– Cotreatment	75 (45)	0
– Bevacizumab	69 (92)	0
– Other drug	6 (8)	0
Overall survival (median in months)	24	15

locus [9–14] as well as in ATP-binding cassette (ABC) transporter genes [15–19] and in the *SLCO1B1* gene [19,20], have been shown to impact clinical outcomes of patients by affecting drug exposure and action.

To improve our knowledge of genetic factors that can contribute to patients' survival, we systematically analyzed the association of common polymorphisms in 11 candidate genes related to irinotecan pharmacokinetics and pharmacodynamics (*ABCB1*, *ABCC1*, *ABCC2*, *ABCC5*, *ABCG1*, *ABCG2*, *SLCO1B1*, *CES1*, *CES2*, *TOP1* and *UGT1*). Our study was based on a well-characterized population of mCRC patients initiating a first-line regimen with FOLFIRI (combining folinic acid, 5-fluorouracil and irinotecan). Positive markers were replicated in an independent larger cohort, and the combined effect of positive markers on survival was assessed in the combined cohort. The biological plausibility was reinforced by functional investigations for validated markers.

Patients & methods

Patient cohorts & clinical outcomes

A prospective cohort of 167 mCRC patients from eastern Canada was recruited from 2003 to 2012 and was set as the discovery cohort. All patients received first-line FOLFIRI and 75 patients also received the monoclonal antibody bevacizumab or another drug. Details on the treatment procedure and eligibility criteria were reported previously [21]. The replication cohort was comprised of 250 mCRC patients treated with FOLFIRI recruited in northeast Italy between 2002 and 2005 [22]. For both cohorts, genomic DNA was extracted from patients' blood as described [21,22]. The main characteristics of the study populations are summarized in Table 1. Overall survival (OS) was defined as the time (in months) between the beginning of irinotecan treatment and death from any cause. This study was conducted in accordance with The Declaration of Helsinki. All participants provided a written informed consent for genetic analysis. The CHU de Quebec ethics committee and the Comitato Etico Indipendente – Centro di Riferimento Oncologico di Aviano approved the research protocols (2012-245). Additional publicly available datasets were used for expression analysis and were comprised of mCRC cases from The Cancer Genome Atlas (TCGA, <https://cancergenome.nih.gov/>) accessed through the Genomic Data Commons Data Portal (<https://portal.gdc.cancer.gov/>) and retrieved with TCGAbiolinks package (version 2.9.5). We also used GSE39582 obtained through the Gene Expression Omnibus (GEO) database with GEOquery package (version 2.48.0).

Genetic analysis

Haplotype-tagging SNPs (htSNPs) were selected in *ABCB1*, *ABCC1*, *ABCC2*, *ABCC5*, *ABCG1*, *ABCG2*, *SLCO1B1*, *CES1*, *CES2*, *TOP1* and *UGT1* genes and their flanking regions (± 5 kb). They were genotyped in both cohorts to maximize the coverage of the genetic variability of the candidate genes, as described [15,23,24]. The list of the htSNPs is provided in Supplementary Table 1. For each htSNP, deviation from Hardy–Weinberg equilibrium was determined using PLINK v1.07 and those deviating from the equilibrium ($p < 0.05$) were not retained for further analysis [25]. Genotypic frequencies of htSNPs in different populations from 1000 Genomes Phase III were retrieved through Ensembl GRCh37 release 91. Prioritization of potential functional markers was established using RegulomeDB and those with a score ≥ 2 were retained for luciferase reporter gene assay (Supplementary Figure 1) [26].

Luciferase reporter assays

Fragments containing the major allele of the SNPs of interest were PCR amplified from genomic DNA using Phusion High-Fidelity DNA polymerase (New England Biolabs, ON, Canada). Fragments were then inserted between SacI and XhoI restriction sites of pGL4.23 luciferase vector (Promega, WI, USA). Constructs carrying minor alleles were obtained either from PCR amplification from genomic DNA or by site-directed mutagenesis using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs). All primers used are listed in Supplementary Table 2 and all constructs were sequenced using the RV primer 3. Cells were seeded in 24-well plates at a density of 1×10^5 cells/well (HepG2, HuH7 and HT-29) or 1.5×10^5 cells/wells (HCT116). After 24 h, cells were transfected with the appropriate construct (200 ng for HCT116, 400 ng for HepG2 and HuH7 and 800 ng for HT-29) and a CMV-renilla internal control construct (2 ng for HCT-116, 4 ng for HepG2 and HuH7 and 20 ng for HT-29) using lipofectamine 2000 as recommended per the manufacturer (Invitrogen, CA, USA). Medium was replaced with fresh medium 3 h post-transfection. Cells were washed with PBS and processed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, WI, USA) 24 h post-transfection. For each condition, luciferase activity was divided by the renilla internal control intensity and reported relative to the ratio obtained for the control (empty pGL4.23 vector). Three independent experiments were performed in triplicate. Differences between groups were compared using a two-tailed t-test performed in GraphPad Prism 5 software (GraphPad Software Inc., CA, USA).

Statistical analysis

Genetic associations between genotypes and OS were tested using Cox proportional hazards models adjusted for covariates (age and cotreatment for the Canadian and combined cohorts, age in the Italian cohort). Additive, dominant and recessive models were fitted independently for each htSNP. Only htSNPs associated with the outcome ($p < 0.1$) in the Canadian cohort were replicated in the Italian cohort. A htSNP was considered validated if associated ($p < 0.05$) with the outcome in the Italian cohort using the same genetic model. A bootstrap resampling (1000 random samples) was used to validate the reliability of the Cox models in the Canadian, the Italian and the combined cohorts. Statistical analyses were performed using SAS v9.4 and IBM SPSS Statistics version 23. Kaplan–Meier analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.) and survival curves were compared using the Log-rank test.

Results & discussion

We systematically analyzed 285 htSNPs of 11 candidate genes in two independent cohorts of 417 mCRC patients receiving first-line FOLFIRI treatment. In the discovery cohort, 54 htSNPs were associated ($p < 0.1$) with OS. Of those, three were validated ($p < 0.05$) in the Italian cohort (Figure 1A). These three markers are located in intronic regions (Supplementary Figure 1) and their frequency varied greatly among ethnic groups (Supplementary Figure 2).

A first marker, *CES1* rs9921399T>C, was associated with an increased OS using a recessive model with adjusted hazard ratios (HR) of 0.53 ($p = 0.069$) in the Canadian cohort, 0.14 ($p = 0.049$) in the Italian cohort and 0.40 ($p = 0.005$) in the combined cohorts (Figure 1A). Kaplan–Meier analysis revealed that carriers of *CES1* rs9921399CC genotype had an improved median OS by 17 months (39 vs 22 months; $p = 0.004$; Figure 1B). *CES1* predominates in the liver whereas *CES2* is also expressed in other tissues such as the small intestine [27,28]. Despite the fact that *CES1* has an inferior affinity for conversion of irinotecan pro-drug into SN-38 compared with *CES2*, reports suggest that *CES1* would account for up to half of this reaction in the liver due to its higher expression levels [29,30]. Our functional investigation of the intronic variant *CES1* rs12443580T>C tightly linked to the intronic rs9921399T>C did not reveal significant differences in transcriptional activity in the condition tested (Supplementary Figure 3A). However, a previous study supports a gene–dose effect for *CES1* on SN-38 formation and exposure in irinotecan-treated Japanese cancer patients [31].

A second marker, the rs17501011G>A located in the first intron of *ABCC1*, was associated with a reduced OS using a dominant model with HR of 1.83 ($p = 0.070$), 2.53 ($p = 0.013$) and 2.08 ($p = 0.003$) in the discovery, replication and combined cohorts, respectively (Figure 1A). Kaplan–Meier analysis showed that carriers of *ABCC1* rs17501011A had a reduced median OS by 6 months (17 vs 23 months; $p = 0.007$; Figure 1C). We further demonstrated that the intronic *ABCC1* rs17501011A allele was associated with a significantly lower reporter gene activity compared with the G allele, in liver cells HepG2 ($p < 0.01$) and HuH7 ($p < 0.01$) and colon cancer cells HCT-116 ($p < 0.05$) and HT-29 ($p < 0.05$), supporting a functional impact (Figure 2A). Few genetic variations

Figure 1. Polymorphisms associated with overall survival of metastatic colorectal cancer patients. (A) Cox proportional hazards models were adjusted for age and cotreatment in Canadian and combined cohorts, adjusted for age in Italian cohort. **(B–D)** Kaplan–Meier survival curves and log-rank p values for *CES1* rs9921399, *ABCC1* rs17501011 and *UGT1* rs1113193. Comb: Combined cohorts; HR_{adj}: Adjusted hazard ratio.

in *ABCC1* were previously linked to increased SN-38 exposure, namely rs6498588A>T located in the 5'-flanking region, and rs35605T>C in exon 13 leading to a synonymous substitution [32,33]. The latter was further reported to contribute to nearly 30% of plasma levels of SN-38 along with *ABCB1* rs10276036 and *UGT1A1**93 [33]. We further exposed that low tumor *ABCC1* expression was associated with shorter survival of mCRC cases from two independent datasets, the TCGA cohort (n = 87; p = 0.013) and GSE39582 (n = 60; p = 0.001) (Figure 2B & C). *ABCC1* encodes the MRP1 transporter, which was involved in drug resistance and treatment failure in multiple cancers [34,35]. Here, we observed shorter survival for patients with lower *ABCC1* tumoral expression and carriers of the rs17501011A allele associated with reduced reporter gene expression. The decreased survival of mCRC cases with lower expression of *ABCC1* may result from several mechanisms. Although expressed at much lower levels in liver compared with other organs such as kidney or intestine, MRP1 is involved in the efflux of multiple endogenous compounds such as glutathione and xenobiotics including SN-38 [36–39]. First, lower *ABCC1* expression in the liver may be associated with reduced efflux to systemic circulation. MRP1 is also involved in the efflux of glutathione and reduced level of glutathione were linked to an increased sensitivity of colon cancer cells to 5-FU [40]. Thus, a reduced expression of MRP1 may increase glutathione levels and limit 5-FU efficacy. Similarly, high *ABCC1* expression in tumors was associated with longer survival in stage IV head and neck squamous cell carcinoma patients [41]. Also, locally advanced esophageal adenocarcinoma patients with high pretherapeutic *ABCC1* tumor expression were found to better respond to neoadjuvant chemotherapy combining 5-FU and cisplatin with or

Figure 2. Functional impact of the *ABCC1* rs17501011 and prognostic value of *ABCC1* gene expression. (A) The *ABCC1* rs17501011A alters transcriptional activity in liver cancer cells HepG2 and HuH7 as well as in colon cancer cells HCT116 and HT-29. (B–C) Influence of *ABCC1* colorectal tumor expression on survival of mCRC patients from the TCGA (n = 87) and the GSE39582 (n = 60) datasets.

*p < 0.05; **p < 0.01.

mCRC: Metastatic colorectal cancer

without paclitaxel [42]. Finally, higher expression of *ABCC1* was shown to promote tumor cell death by increasing oxidative stress through a reduction of intracellular glutathione levels [43].

A third variant, *UGT1* rs1113193G>A, was linked to shorter survival using a recessive model with HR of 2.00 (p = 0.040), 2.47 (p = 0.032) and 2.12 (p = 0.004) in the discovery, replication and combined cohorts (Figure 1A). Kaplan–Meier showed that homozygous carriers of the rs1113193A allele had a reduced OS by 10 months (13 vs 23 months; p = 0.005; Figure 1D). The reporter gene assays with rs1113193G>A and its tightly linked rs7608713G>A variant did not reveal change of gene expression in the condition tested (Supplementary Figure 3B & C). The *UGT1* locus encodes nine UGT1A functional enzymes including UGT1A1 conjugating bilirubin, also responsible for the glucuronidation of SN-38 [44–47]. Supportive of a potential functional impact of this marker or a linked variant, the *UGT1* rs1113193G>A was associated with altered bilirubin level in individuals of the Framingham Heart Study (European descent subjects) and in a study combining individuals from the Framingham Heart Study, the Rotterdam study and the Age, Gene, Environment and Susceptibility – Reykjavik study [48,49].

Figure 3. Co-occurrence of markers linked to reduced survival. (A) Carriers of several markers had a reduced overall survival compared with the reference group carrying only one marker. **(B)** Haplotype analysis confirmed that carriers of multiple markers had a shorter overall survival. Genotypes in yellow are associated with reduced survival, those in green to longer survival. Patients of the Canadian and Italian cohorts were combined. Only genotype combinations carried by at least three patients were conserved. HR_{adj}: Adjusted hazard ratio.

Finally, we conducted a combined analysis of *CES1* rs9921399, *ABCC1* rs17501011 and *UGT1* rs1113193 markers and included a marker (*RPL28* rs4806668) recently reported by our group to be associated with survival of mCRC patients [50]. In the combined cohort, carriers of two or three unfavorable genotypes displayed shorter survival with HR values of 2.17 ($p < 0.001$) and 5.35 ($p = 0.004$), respectively, compared with carriers of one unfavorable genotype, whereas carriers of all favorable genotypes experienced prolonged survival (Figure 3A). A haplotype analysis confirmed an enhanced effect of these unfavorable markers on survival with HR values of 1.98 ($p = 0.038$) to 2.97 ($p = 0.018$) for carriers of unfavorable genotypes, whereas HR for noncarriers was 0.31 ($p = 0.002$) as shown in Figure 3B.

Further studies are needed to prospectively replicate these associations. Several patients of the discovery cohort received FOLFIRI in combination with another drug (mainly bevacizumab), which represents a limitation of this study. Another limitation is the fact that we did not perform correction for multiple testing given the limited number of patients in each cohort. However, markers were investigated in two independent cohorts, thus reducing the possibility of false-positive associations. We conclude that the combined analysis of polymorphisms in genes related to irinotecan pharmacology may eventually help predict survival of mCRC patients.

Executive summary

- We performed a systematic analysis of 285 haplotype-tagging SNPs in 11 genes related to irinotecan pharmacology in relation to overall survival (OS) in two independent cohorts of 417 metastatic colorectal cancer (mCRC) patients receiving first-line FOLFIRI treatment.
- *CES1* rs9921399T>C was predictive of prolonged OS, whereas *ABCC1* rs17501011G>A and *UGT1* rs1113193G>A were associated with reduced OS.
- *ABCC1* rs17501011A allele caused reduced reporter-gene activity in liver ($p < 0.01$) and colon ($p < 0.05$) cells, whereas lower *ABCC1* tumor expression in mCRC patients was associated with reduced OS in two independent cohorts.
- A haplotype analysis supports a combined effect of these polymorphisms on survival in mCRC patients.
- This study identifies common variants in irinotecan-related pathways that may contribute to survival of patients with colorectal cancer and requires further validation.

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