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Companion Diagnostics and Cancer Biomarkers

Molecular Cancer Therapeutics

Pharmacogenetic Predictors of Outcome in Patients with Stage II and III Colon Cancer Treated with Oxaliplatin and Fluoropyrimidine-Based Adjuvant Chemotherapy

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Abstract

Identifying molecular markers for tumor recurrence is critical in successfully selecting patients with colon cancer who are more likely to benefit from adjuvant chemotherapy. We investigated the effect of single-nucleotide polymorphisms (SNP) within genes involved in oxaliplatin and fluoropyrimidines metabolism, DNA repair mechanisms, drug transport, or angiogenesis pathways on outcome for patients with stage II and III colon cancer treated with adjuvant chemotherapy. Genomic DNA was extracted from formalin-fixed paraffin-embedded samples of 202 patients with stage II and III colon cancer receiving oxaliplatin-based adjuvant chemotherapy from January 2004 to December 2009. Genotyping was performed for 67 SNPs in 32 genes using the MassARRAY (SEQUENOM) technology. Our results were validated in an independent cohort of 177 patients treated with the same chemotherapy regimens. The combination of the *selectin E* (*SELE*) rs3917412 G>A G/G and the *methylentetrahydrofolate reductase* (*MTHFR*) rs1801133 T/T genotypes was associated with a significantly increased risk for recurrence in both the training [RR = 4.103; 95% confidence interval (CI), 1.803–9.334; $P = 0.001$] and the validation cohorts (RR = 3.567; 95% CI, 1.253–10.151; $P = 0.017$) in the multiple regression analysis considering the stage, lymphovascular invasion, and bowel perforation as covariates. The combined analysis of these polymorphisms was also significantly associated with overall survival in both cohorts (RR = 3.388; 95% CI, 0.988–11.623; $P = 0.052$, and RR = 3.929; 95% CI, 1.144–13.485; $P = 0.020$, respectively). Our findings suggest that the *SELE* rs3917412 and *MTHFR* rs1801133 SNPs could serve as pharmacogenetic predictors of tumor recurrence in patients with early-stage colon cancer treated with oxaliplatin-based adjuvant chemotherapy, thus allowing personalized selection of treatment to optimize clinical outcomes. *Mol Cancer Ther; 13(9); 2226–37. ©2014 AACR.*

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Introduction

Oxaliplatin- and fluoropyrimidine-based chemotherapy is currently recommended by the main clinical practice guidelines as the standard adjuvant treatment for completely resected stage III and selected high-risk stage II colon cancer. Its benefits have been most clearly demonstrated in stage III disease, with an approximately 30% reduction in the risk of recurrence and a 22% to 32% reduction in mortality (1–3). The value of adjuvant chemotherapy in stage II disease remains controversial and data from randomized trials and meta-analyses indicate that, if there is a benefit for fluoropyrimidine-based chemotherapy in those patients, it does not exceed an absolute improvement in 5-year survival of 5% (4, 5).

Conventional clinicopathological risk factors such as staging, grade, or tumor histology have been used as the standard measure of prognosis for many years. However, these factors do not clearly distinguish between patients who have a high or low risk of disease recurrence, and do not predict which patients are likely to benefit from

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chemotherapy. In current practice, the majority of patients with stage II and III colon cancer receive treatment unnecessarily, either because they were cured with surgery alone or because they will relapse despite adjuvant treatment. It is therefore essential to improve prognostication beyond that achieved by the current tumor–node–metastasis (TNM) staging system and to develop reliable molecular markers to stratify patients with early-stage colon cancer for risk of relapse, which in turn would allow treatment options to be tailored to the individual, spare other needless toxicity and the financial burden of chemotherapy that will not work.

Several potential molecular predictors of recurrence risk and chemotherapy benefit have been investigated (microsatellite instability, KRAS expression, p53 expression, gene, or microRNA signatures), but they still require validation and are not part of standard clinical practice (6–9). More recently, the necessary interactions between tumor and host in the oncological process have raised questions about how the individual genomic biology influences cancer progression and response to chemotherapy, consequently generating a great amount of critical data. In the last few years, several genetic polymorphisms within genes involved in the metabolism, transport, and detoxification of fluoropyrimidines and oxaliplatin have been considered in an attempt to optimize treatment in patients with colon cancer (10–18), but, up to now, their clinical applications remain limited. Furthermore, numerous variants have been identified in genes related to the DNA-repair complex, angiogenesis, and other relevant pathways that may alter their expression and/or activity, thereby causing interindividual differences in tumor recurrence capacity and chemoresistance (18–22).

Many of the recently published studies have used a methodology based on one or a few single-nucleotide polymorphisms (SNP). Although this approach could be useful, it is necessary to previously know the SNPs and the function of the genes involved, which has often generated contradictory outcomes. In addition, because chemotherapy affects multiple targets, there is little likelihood that a single SNP could account for the variability of the treatment outcomes. Conversely, with the recent advance in molecular genotyping technology, the genotyping of a large number of SNPs involved in the different stages that take part in the triggering of drug activation, metabolisms, and target expression is now possible. This new approach seems to have a greater capability in examining genetic differences in individual patients with regard to drug response or side effects (18, 23).

In this study, we performed a comprehensive analysis to evaluate associations between a panel of SNPs within candidate genes known or suspected to be involved, based on previously described associations or putative functional effects, in oxaliplatin and fluoropyrimidine pathways with clinical outcome in a cohort of patients with early-stage colon cancer treated with adjuvant chemotherapy. In addition, we have explored whether SNPs with significant prognostic value showed stage- or gender-specific differences and if such differences correlated with clinical outcomes.

Materials and Methods

Study protocol and design, treatment, and evaluations

Patients with high-risk stage II and III colon cancer treated at 2 Spanish institutions in Madrid (La Paz University Hospital and Puerta de Hierro Majadahonda University Hospital) between January 2004 and December 2009 were eligible for our study. The study protocol specified the inclusion criteria as follows: (i) completely resected colon adenocarcinoma located at $\geq$15 cm of the anal verge as determined by endoscopy or above the peritoneal reflection in the surgical resection without any evidence of metastatic disease; (ii) stage III or high-risk stage II colon cancer with at least one of the following clinicopathological features: T4 primary, inadequate lymph node sampling (less than 14 lymph nodes at the time of surgery), tumor presentation with bowel obstruction or perforation, lymphovascular or perineural invasion, poorly differentiated histology, or a high preoperative serum carcinoembryonic antigen (CEA) level; (iii) oxaliplatin- and fluoropyrimidine-based adjuvant chemotherapy regimen within 6 to 8 weeks of surgery. Main exclusion criteria were as follows: (i) macroscopic or microscopic evidence of residual tumor in the surgical specimen; (ii) any prior adjuvant or neoadjuvant radiotherapy; (iii) severe renal or hepatic disorder, bone marrow suppression, or disabling peripheral neuropathy; (iv) history of other malignancies within the previous 5 years, except curatively treated cervix carcinoma *in situ* or basal cell carcinoma of the skin. To validate our results, we used an independent cohort of patients with high-risk stage II and III colon cancer treated with the same chemotherapy regimens at 4 different Institutions in Valencia (La Fe University Hospital, Valencian Oncology Institute), Alicante (Elche General University Hospital), and Barcelona (Clinic University Hospital) at the same time period.

All patients were treated with oxaliplatin-based adjuvant chemotherapy (12 cycles of FOLFOX or 8 cycles of CAPOX regimens) as determined by each responsible physician according to the patient characteristics or local practice guidelines.

Before adjuvant treatment, patients were medically assessed and baseline demographic and pathologic variables (age, sex, site of disease, tumor grade, T-stage, N-stage, number of resected nodes, lymphovascular or perineural invasion, bowel obstruction or perforation, preoperative serum CEA level, and chemotherapy regimen) were recorded by reviewing medical histories and pathologic tumor samples. The staging of colorectal cancer was assessed according to the TNM classifications set out by the American Joint Committee on Cancer (AJCC). All patients were included in the colon cancer surveillance program of each attending institution providing the following information: history,

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physical examination, and CEA determination every 3 months for 2 years and every 6 months at years 3, 4, and 5 after surgery; computed tomography scans of chest and abdomen every 6 months; and colonoscopy at year 1 and thereafter every 3 to 5 years.

The protocol was approved by the local Ethic Committees and all participants signed informed consent for the analysis of molecular correlates.

Selection of candidate polymorphisms

Common and putatively functional SNPs within genes involved in the metabolism and detoxification of oxaliplatin and fluoropyrimidines, DNA repair mechanisms, drug transport, *epidermal growth factor receptor (EGFR)*, and *vascular endothelial growth factor receptor (VEGF)*–dependent and –independent angiogenesis pathways have been selected on the basis of the public literature resources and databases. Haplotype-tagging SNP and additional informative SNP were selected from the International Haplotype Mapping (HapMap; www.hapmap.org) and from the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/SNP), respectively.

We used the following criteria to select genes for study: (i) credible scientific basis to support a gene's involvement in the previously referred pathways; (ii) genes with an established, well-documented genetic polymorphism that could alter its function in a biologically relevant manner [either published data or predicted function using Functional SNP (F-SNP) database, http://compbio.cs.queensu.ca/F-SNP]; and (iii) minor allelic frequency of 5% or more in Caucasians (for relative allelic frequencies of the polymorphisms in different ethnicities, we refer to the population genetic section in the Ensembl Genome Browser: http://uswest.ensembl.org/index.html). Overall, 67 SNPs in 32 genes were analyzed (Table 1).

Polymorphisms genotyping

Tumor samples and corresponding adjacent normal mucosa tissues from the study patients were obtained at the time of surgery. For the SNP analysis, tumor sections were selected from formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks containing at least 80% of tumor tissue. Genomic DNA was extracted using the "MasterPure Complete DNA Purification Kit" (Epicentre Biotechnologies) according to manufacturer's instructions.

Genotyping analysis was performed using the SEQUENOM MassARRAY platform [National Genotyping Center (CeGen), Santiago de Compostela, Spain]. Genotyping process consists of 2 reactions. In the first one, DNA fragments containing the SNP of interest are amplified using a multiplex polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique. In the second one, the allelic discrimination of single base extension (SBE) reaction is performed. The primer hybridizes near the polymorphic site being genotyped and extends a base according to the sequence of the variant site. Through the use of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, the mass of the extended primer is determined. The end result is a mass spectrum of the multiple products obtained using extension primers of different lengths in a single reaction, which generates sufficiently spaced groups of peaks. Then, the analysis software assigns genotypes to SNPs in each trial based on the masses obtained. The peak corresponding to the primers that have not extended is used as a control to determine the effectiveness of each extension reaction. The calculation of the ratios of the peaks of each allele generates a report with the cluster analysis (24, 25).

Statistical analysis

The primary endpoint of this study was the relationship between SNPs and disease-free survival (DFS). The secondary endpoint was the relationship between SNPs and overall survival (OS). DFS was computed from the date of intended curative resection to the date of the first documented tumor recurrence. DFS was censored at the date of death or at the last follow-up for disease-free patients. OS was defined as the time from the surgery until death from any cause.

The genotypes for each SNP were analyzed as a 3-group categorical variable (reference model). Deviation from Hardy–Weinberg equilibrium (HWE) for allelic distributions of all SNPs was tested using a χ^2 test. We removed SNPs that deviated from HWE in the validation analysis. The distribution of SNPs across baseline demographic, clinical, and pathologic characteristics was examined using the Mann–Whitney test for continuous variables and the χ^2 test or Fisher exact test for categorical variables.

The DFS and OS estimates were calculated using Kaplan–Meier curves. The associations between clinicopathological characteristics and SNPs with DFS and OS were assessed using a univariate survival analysis based on the log-rank test. The Cox proportional hazards regression model was used to evaluate the independent effects of the SNPs on DFS and OS using the clinicopathological data and the chemotherapy regimen as covariates. Genotypes were grouped after trying to seek the most significant association with phenotypes. Two-sided P values <0.05 were considered statistically significant. All statistical analyses were carried out using the SAS statistical package software version 9.2 (SAS Institute Inc.).

Results

Patient characteristics and survival

A total of 405 patients were registered onto the study between January 2004 and December 2009. The genotyping analysis was possible in tumor samples from 202 of the 221 (91.4%) patients included as the training cohort and in 177 of the 184 (96.19%) patients included as the validation cohort. The median follow-up was 51.4 months (range, 7–96) in the training set and 49.7 months (range, 9–82) in the validation set. Detailed clinicopathological characteristics of the patients included in the analysis are listed in Table 2. The incidence of grade 2 tumors,

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Table 1. Analyzed SNPs

Category	Gene	SNPs
Metabolisms/ detoxification of fluoropyrimidines	*Thymidylate synthase* (*TYMS*)	rs34743033, rs2244500
	Dihidropyrimidine dehydrogenase (*DPYD*)	rs3918290, rs6663828, rs970337
	Methylentetrahydrofolate reductase (*MTHFR*)	rs1801133, rs1801131, rs6541003
	Orotate phosphoribosyltransferase (*OPRT*)	rs1801019, rs3772807
Metabolisms/ detoxification of oxaliplatin and DNA repair genes	*Glutatione S-transferase pi 1* (*GSTP1*)	rs1695, rs749174
	Excision repair cross-complementing 1 (*ERCC1*)	rs11615, rs 3212964
	Excision repair cross-complementing 2 (*ERCC2/XPD*)	rs1799793, rs 238404, rs13181
	Excision repair cross-complementing 5 (*ERCC5/XPG*)	rs4150279, rs4150360
	Excision repair cross-complementing 6 (*ERCC6*)	rs2228527, rs7907557
	X-ray repair cross-complementing group 1 (*XRCC 1*)	rs25487, rs25489, rs12611088, rs3213255
	X-ray repair cross-complementing group 2 (*XRCC 2*)	rs3218536,rs 3218408, rs3111417
	RAD23 homolog B (*RAD23B*)	rs2147072, rs10759225, rs1805329
	Methylguanine-DNA methyltransferase (*MGMT*)	rs1803965, rs656639
	Xeroderma pigmentosum, complementation group A (*XPA*)	rs3176751, rs3176639
	Xeroderma pigmentosum, complementation group C (*XPC*)	rs2733534
EGFR pathway	*Epidermal growth factor* (*EGF*)	rs6824594, rs929446
	Epidermal growth factor receptor (*EGFR*)	rs11543848
	Akt murine thymoma viral oncogene homolog 1 (*AKT1*)	rs3803304, rs10142069, rs3001371
	Akt murine thymoma viral oncogene homolog 2 (*AKT2*)	rs2304186, rs7260517
Angiogenesis	*Vascular endothelial growth factor* (*VEGF*)	rs833070, rs3025039
	Interleukin 8 (*IL8*)	rs4073, rs2227306
Others	*ATP-binding cassette, subfamily B, member 1* (*ABCB1*)	rs1045642
	ATP-binding cassette, subfamily G, member 2 (*ABCG2*)	rs2231142, rs2728124, rs3114018
	Sulfotransferase (*SULT1A1*)	rs1968752
	Selectin E (*SELE*)	rs5361, rs3917412, rs3917436
	Matrix metalloproteinase 1 (*MMP1*)	rs498186
	Matrix metalloproteinase 3 (*MMP3*)	rs602128
	Cyclin H (*CCNH*)	rs2230641, rs3093816
	Intercellular adhesion molecule 1 (*ICAM1*)	rs3093030
	Insulin-like growth factor 1 receptor (*IGF1R*)	rs2229765, rs939626
	Mechanistic target of rapamycin (*MTOR*)	rs2295080, rs357278, rs6895953
	LSM3 homolog, U6 small nuclear RNA associated (*LSM3*)	rs2607739

perineural invasion, and bowel perforation was significantly higher in the training cohort than in the validation cohort. The 74.8% of patients in the training set were treated with the CAPOX regimen and the 25.2% with FOLFOX, whereas most patients (87.6%) received FOLFOX adjuvant chemotherapy in the validation cohort. No statistically significant differences with respect to other variables were found between both cohorts.

In the training set, 63 (31.18%) patients had experienced tumor recurrence and the 3-year DFS was 72.2% [95% confidence interval (CI), 69–75.4]. Thirty-one patients (15.34%) died and 3-year OS was 89.1% (95% CI, 86.9–91.3). Patients with stage III [hazard ratio (HR), 3.86; 95% CI, 1.78–8.37; $P = 0.001$], lymphovascular invasion (HR, 2.15; 95% CI, 1.28–3.59; $P = 0.003$) and bowel perforation (HR, 1.89; 95% CI, 1.034–3.46; $P = 0.039$) were more likely to develop tumor recurrence in the multivariate analysis considering the most relevant demographic and clinicopathological characteristics as covariates. We did not observe any significant associations between other variables and DFS. In the validation set, 49 (27.68%) patients had a tumor recurrence, and the 3-year DFS was 75.6% (95% CI, 70.1–82.9). Twenty-seven patients (15.25%) died and 3-year OS was 87.4% (95% CI, 85–89.8).

SNP associated with survival

Genotyping was successful in at least 90% of cases for each SNP analyzed in tumor tissue, with the exception of *X-ray cross-complementing gene-1* (*XRCC1*) rs25487 (82.06%) and *thymidylate synthase* (*TYMS*) rs34743033 (81.54%). In failed cases, genotyping was not successful because of limited quantity of extracted genomic DNA. The allelic frequencies for all polymorphisms were within the probability limits of HWE, with the exception of

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Table 2. Clinical and pathologic characteristics in the training and validation cohorts

Characteristics	All patients ($n = 379$)	Training set ($n = 202$)	Validation set ($n = 177$)	*P*
Median age (range)	61.94 (23–85)	63.82 (23–85)	59.8 (23–76)	0.231
Sex				
Men	194 (51.18%)	115 (56.93%)	79 (44.63%)	0.068
Women	185 (48.81%)	87 (43.06%)	98 (55.36%)	
Tumor site				
Cecum-right colon	115 (30.34%)	60 (29.7%)	55 (33.07%)	0.235
Transverse colon	23 (6.06%)	15 (7.42%)	8 (4.51%)	
Left colon	52 (13.72%)	28 (13.86%)	24 (13.55%)	
Sigma	189 (49.86%)	99 (49%)	90 (50.84%)	
Differentiation				
Grade 1	60 (15.83%)	19 (9.4%)	41 (23.16%)	0.001
Grade 2	274 (72.29%)	160 (79.2%)	114 (64.4%)	
Grade 3	45 (11.87%)	23 (11.4%)	22 (12.42%)	
Stage				
II	105 (27.7%)	60 (29.7%)	45 (25.42%)	0.208
III	274 (72.3%)	142 (70.3%)	132 (74.57%)	
Lymphovascular invasion				
Yes	164 (43.27%)	88 (43.56%)	76 (42.93%)	0.311
No	215 (56.73%)	114 (56.43%)	101 (57.06%)	
Perineural invasion				
Yes	119 (31.39%)	73 (36.13%)	46 (25.98%)	0.025
No	260 (68.61%)	129 (63.86%)	131 (74.01%)	
Bowel obstruction				
Yes	89 (23.48%)	53 (26.23%)	36 (20.33%)	0.184
No	290 (76.51%)	149 (73.76%)	141 (79.66%)	
Bowel perforation				
Yes	61 (16.1%)	41 (20.3%)	20 (11.29%)	0.012
No	318 (83.9%)	161 (79.7%)	157 (88.71%)	
Type of adjuvant CT				
FOLFOX	206 (54.4%)	51 (25.24%)	155 (87.57%)	0.001
CAPOX	173 (45.6%)	151 (74.75%)	22 (12.43%)	

RAD23B rs1805329 (data not shown). Concordance between genotyping in tumor samples and adjacent normal mucosa tissue was found in the 95% to 99% of analyzed SNPs. There were no significant associations between the SNPs and baseline demographic, clinical, or pathologic characteristics (data not shown).

In the training cohort, *selectin E* (*SELE*) rs3917412 G>A and *methylenetetrahydrofolate reductase* (*MTHFR*) rs1801133 C>T SNPs were significantly associated with DFS in the univariate analysis. A significant association was also found in the logistic regression analysis using the stage, lymphovascular invasion, and bowel perforation as covariates. A total of 111 patients (56.06%) were homozygous for the G allele (G/G) of the *SELE* rs3917412 G>A SNP, 77 (38.89%) were heterozygous (G/A) and 10 (5.05%) were homozygous for the A allele (A/A; Supplementary Table S1). Patients with the G/G homozygous genotype have a 3-year DFS of 67.6% (95% CI, 63.2%–72%) compared with 80.4% (95% CI, 76.1%–84.7%) in heterozygous or homozygous carriers of the A allele (HR, 0.524; 95% CI, 0.310–0.884; $P = 0.016$; Fig. 1A). In the case of the *MTHFR* rs1801133 C>T SNP, 86 patients (42.57%) were homozygous for the C allele (C/C), 77 (38.12%) were heterozygous (C/T) and 26 (12.87%) were homozygous for the T allele (T/T; Supplementary Table S1). Patients with the T/T homozygous genotype have a 3-year DFS of 61.5% (95% CI, 52%–71%) compared with 74.2% (95% CI, 70.8%–77.4%) in heterozygous or homozygous carriers of the C allele (HR, 1.924; 95% CI, 1.019–3.634; $P = 0.044$; Fig. 1B).

Furthermore, we examined the possible interaction between the *SELE* rs3917412 G>A and *MTHFR* rs1801133 C>T SNPs and outcome. In the combined genotype analysis, patients harboring the *SELE* rs3917412 G/G and *MTHFR* rs1801133 T/T genotypes were more likely to develop tumor recurrence than those displaying the *SELE* rs3917412 any A and/or *MTHFR* rs1801133 any C genotypes (HR, 3.28; 95% CI, 1.46–7.37; $P = 0.004$). The ability to predict tumor recurrence of this combined analysis was independently validated in the second cohort (HR, 3.85; 95% CI, 1.35–10.95; $P = 0.011$). In addition, the presence of

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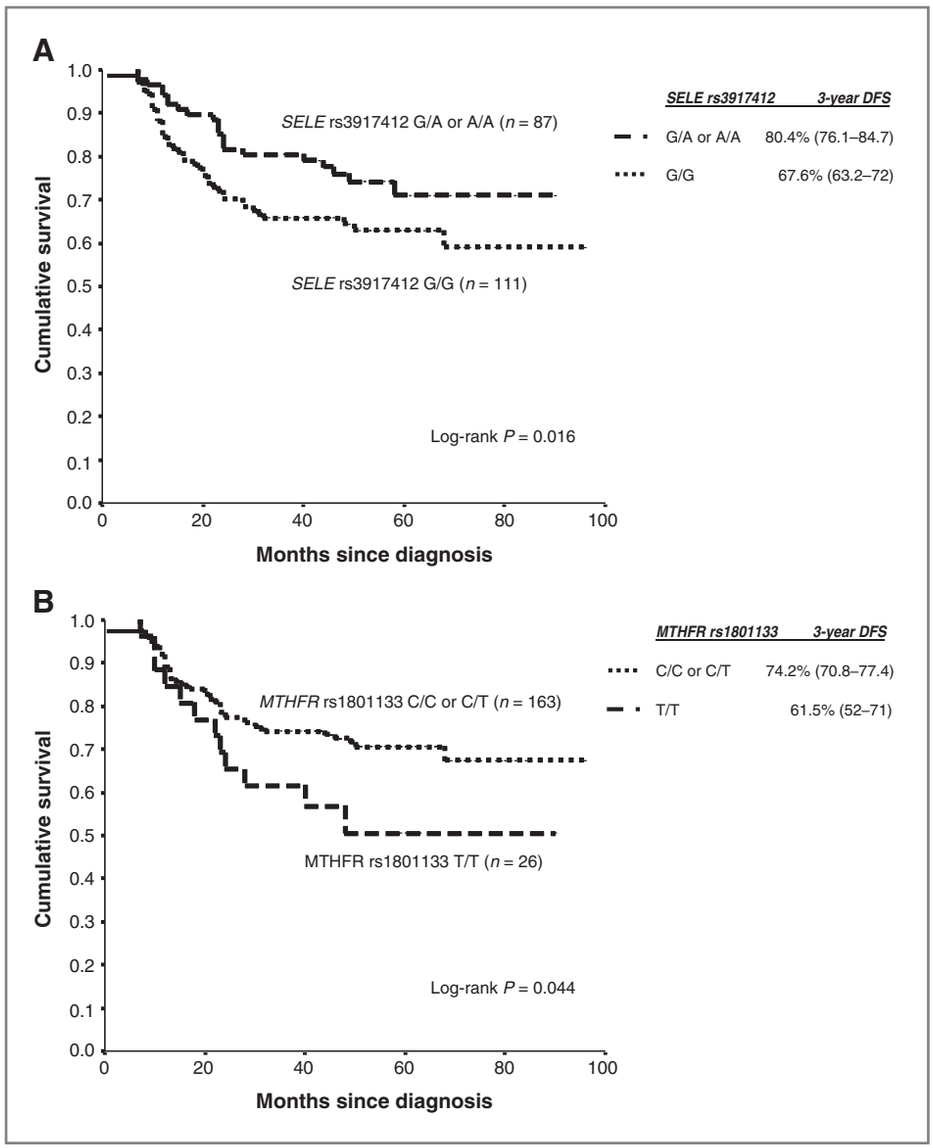

Figure 1. DFS by *SELE* rs3917412 (A) and *MTHFR* rs1801133 (B) SNPs in the training cohort.

the unfavorable haplotype was also significantly associated with shorter DFS in the multivariate analysis using the disease stage, lymphovascular invasion, and bowel perforation as covariates, in the discovery (HR, 4.103; 95% CI, 1.803–9.334; $P = 0.001$) and validation sets (HR, 3.567; 95% CI, 1.253–10.151; $P = 0.017$; Table 3 and Fig. 2). About OS, the combination of the *SELE* rs3917412 G/G and *MTHFR* rs1801133 T/T genotypes was associated with worse outcome in both the training (HR, 3.388; 95% CI, 0.988–11.623; $P = 0.052$) and the validation cohorts (HR, 3.929; 95% CI, 1.144–13.485; $P = 0.028$; Supplementary Table S2 and Fig. 3).

Finally, we have tested whether the *SELE* rs3917412 and *MTHFR* rs1801133 SNPs could be associated with stage- or gender-specific outcome. The allelic distribution of these 2 polymorphisms did not vary significantly between males and females or stage II and III colon cancer (data not shown). When the patient population was separated by stage in the multivariate model, we found that the combination of the *SELE* rs3917412 G/G and *MTHFR* rs1801133 T/T genotypes was significantly associated with DFS in stage III patients in both cohorts. In the training cohort, patients with the *SELE* G/G and *MTHFR* T/T genotype had a shorter 3-year DFS of 50.2% (95% CI, 44%–56.4%) compared with those with the *SELE* any A and *MTHFR* any C (71.9%; 95% CI, 66.2%–77.6%) or *SELE* any A or *MTHFR* any C genotypes (68.3%; 95% CI, 61.5%–75.1%; HR, 2.657; 95% CI, 1.303–5.418; $P = 0.027$). In the validation cohort, patients with the *SELE* G/G and *MTHFR* T/T genotype also had a higher risk of recurrence (3-year DFS, 49.9%; 95% CI, 44%–56.1%) than *SELE* any A and *MTHFR* any C (3-year DFS, 77.8%; 95% CI, 71.2%–84.1%) or *SELE* any A or *MTHFR* any C carriers (3-year DFS, 69.6%; 95% CI, 63.9–75.8; HR, 2.591; 95% CI, 1.023–

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Table 3. Cox regression multivariate analysis for DFS in the training and validation cohorts

	Training cohort			Validation cohort		
	n	HR (95% CI)	*P*	*n*	HR (95% CI)	*P*
Stage			0.001			0.343
Stage II	60 (29.7%)	1 (reference)		45 (25.42%)	1 (reference)	
Stage III	142 (70.3%)	3.03 (1.43–6.43)		132 (74.57%)	1.53 (0.63–3.67)	
Lymphovascular invasion			0.003			<0.001
No	114 (56.43%)	1 (reference)		101 (57.06%)	1 (reference)	
Yes	88 (43.56%)	2.26 (1.31–3.87)		76 (43.94%)	4.67 (2.28–9.55)	
Bowel perforation			0.349			<0.001
No	161 (79.7%)	1 (reference)		157 (88.71%)	1 (reference)	
Yes	41 (20.3%)	1.32 (0.74–2.35)		20 (11.29%)	3.56 (1.88–6.77)	
SELE rs3917412 + *MTHFR* rs1801133			0.003			0.035
2 favorable (SELE G/A-A/A and *MTHFR* C/T-C/C)	71 (38.17%)	1 (reference)		63 (38.41%)	1 (reference)	
1 favorable (SELE G/A-A/A or *MTHFR* C/T-C/C)	100 (53.76%)	1.68 (0.93–3.03)	0.087	92 (56.09%)	1.99 (1.013–3.909)	0.046
2 unfavorable (*SELE* G/G and *MTHFR* T/T)	15 (8.06%)	4.103 (1.803–9.33)	0.001	9 (5.48%)	3.56 (1.25–10.15)	0.017

6.566; $P = 0.045$; Supplementary Table S3). In the case of stage II disease, the *SELE* G/G and *MTHFR* T/T genotype was associated with a nonsignificant trend toward decreased DFS compared with patients with the *SELE* any A and/or *MTHFR* any C genotypes in both the training (HR, 3.93; 95% CI, 0.994–16.478; $P = 0.051$) and the validation cohorts (HR, 4.174; 95% CI, 0.880–21.082; $P = 0.106$). In the sex-specific analysis, female patients harboring the combination of the *SELE* rs3917412 G/G and *MTHFR* rs1801133 T/T genotypes were more likely to develop recurrence than those displaying the *SELE* rs3917412 any A and/or *MTHFR* rs1801133 any C genotypes in the training set (3-year DFS, 72.6% *SELE* any A and *MTHFR* any C, 69.4% *SELE* any A or *MTHFR* any C, 48.9% *SELE* G/G and *MTHFR* T/T; HR, 4.119; 95% CI, 1.466–11.577; $P = 0.007$) and the validation set (3-year DFS, 79.4% *SELE* any A and *MTHFR* any C, 70.2% *SELE* any A or *MTHFR* any C, 49.2% *SELE* G/G and *MTHFR* T/T; HR, 4.322; 95% CI, 0.973–12.32; $P = 0.051$; Supplementary Table S4). However, there were no statistically significant survival differences according to these polymorphisms in male patients.

Discussion

Individualization of adjuvant therapy in colon cancer using robust, evidence-based prediction of efficacy is a highly attractive strategy. To the best of our knowledge, this is the largest pharmacogenomic study performed in a Caucasian cohort of patients with high-risk stage II and III colon cancer who had been homogenously treated with oxaliplatin- and fluoropyrimidine-based adjuvant chemotherapy. We were able to demonstrate that the *SELE* rs3917412 and the *MTHFR* rs1801133 germline SNPs are independently associated with tumor recurrence and could serve as gender- and stage-specific predictors of outcome. In addition, this is the first study in which the association between significant SNPs and patients' outcome has been validated in an independent cohort of patients with stage II and III colon cancer treated with the same chemotherapy regimens.

The prognostic and predictive value of SNPs in genes encoding key enzymes for the metabolism of fluoropyrimidines, such as *TYMS*, *dihydropyrimidine dehydrogenase* (*DPYD*), or *MTHFR*, has been assessed in patients with colon cancer, but the results are often contradictory and inconclusive, particularly in patients treated adjuvantly (10–13, 17, 18, 26). Functional polymorphisms within genes involved in DNA repair pathways or in the metabolism and detoxification of oxaliplatin have also been associated with the efficacy of this agent. Most analysis have been conducted in patients with metastatic disease (12, 15, 16, 18), whereas the pharmacogenetic of oxaliplatin-based adjuvant chemotherapy has not been widely studied (14, 17, 27). In a previous European report including 98 patients with stage III colon cancer, none of the tested SNPs in *glutathione S-transferase pi 1* (*GSTP1*), *excision repair cross-complementing 1* (*ERCC1*), and *excision repair cross-complementing 2* (*ERCC2*) were reliable markers of response to oxaliplatin therapy (14). Furthermore, in contrast to a pharmacogenetic study of adjuvant FOLFOX for Korean patients in which polymorphisms in *XRCC1* and *TYMS* were related to DFS (17), no significant associations between all these SNPs and outcome have been found in our report.

This study, however, is the largest to show that one of the *SELE* SNPs may be an important prognostic marker for colon cancer relapse after oxaliplatin-based adjuvant chemotherapy. E-selectin is a specific cell adhesion molecule expressed on endothelial cells, which is activated by cytokines released during the inflammatory process and involved in adhesion and extravasation of leucocytes

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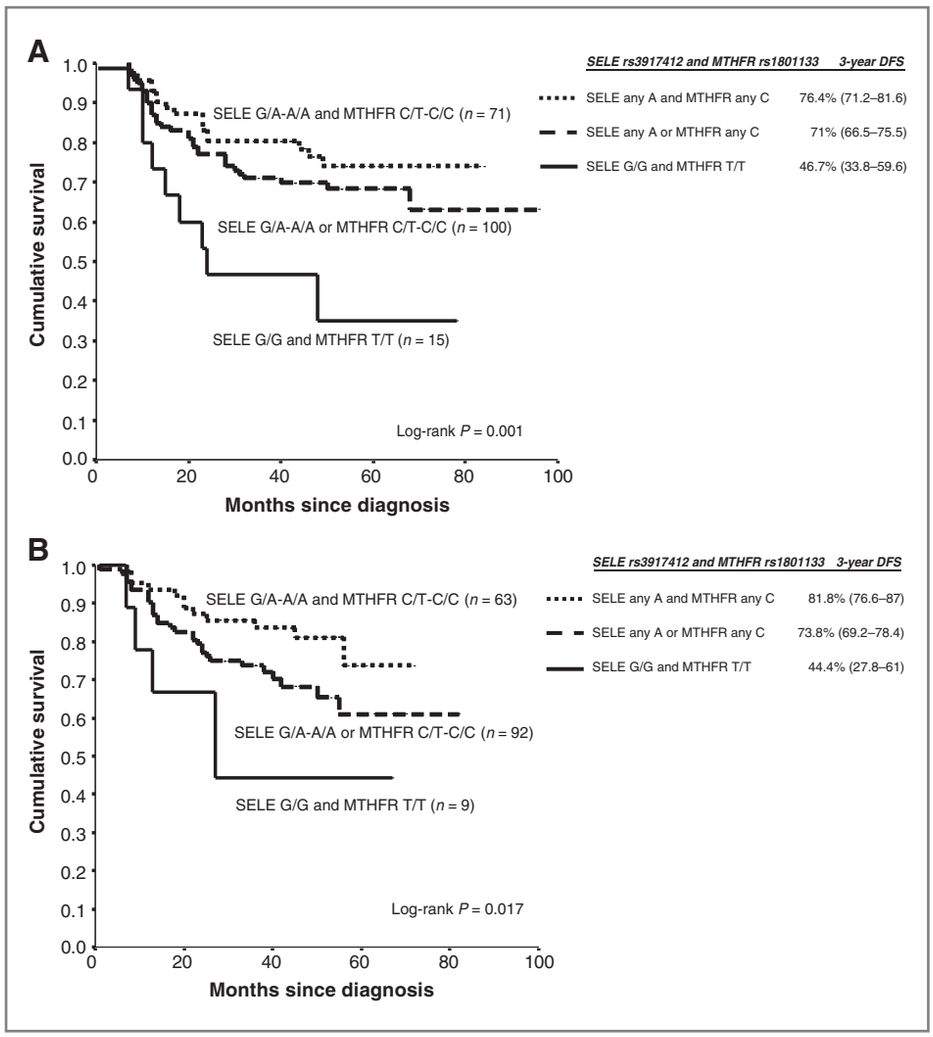

Figure 2. DFS by combination of *SELE* rs3917412 and *MTHFR* rs1801133 SNPs in the training (A) and validation cohorts (B).

carrying the E-selectin ligands (28). Intriguingly, cancer cells hijack the inflammatory system and interact with the vascular endothelium via E-selectin. This interaction confers metastatic properties to colon cancer cells by promoting its extravasation (29), inducing chemotaxis (30, 31) and triggering the activation of the promigratory p38 and prosurvival ERK and PI3K/NF-κB pathways (32). The correlation between the expression of E-selectin and the E-selectin ligands with the metastatic potential of malignant cells has been well documented for colon cancer (20, 30, 33, 34). Several SNPs have been identified within the *SELE* gene. The most common one, *SELE* rs5361, is present in the 10% to 15% of the Caucasian population. It is located in exon 4 and results in the substitution of a serine by an arginine (S128R) within the extracellular domain of the receptor, increasing the adhesion of cancer cells to the endothelium (20, 30). Few studies to date have evaluated the potential prognostic role of *SELE* SNPs in patients with colon cancer. A French report has found that the variant allele of the *SELE* rs5361 SNP was associated with a higher risk of relapse and death in patients with stage II and III colon cancer (20). Although these results could not be confirmed in our series, we have demonstrated, however, the prognostic value of the *SELE* rs3917412 G>A SNP. Curiously, this mutation occurs in nonexpressed intronic regions of the *SELE* gene and its functional consequences are not definitively established. Although the detailed molecular mechanisms involved in how the *SELE* rs3917412 G>A SNP exerts effects on colon cancer cells are unknown and the probability of inducing biological effects on gene expression is higher for polymorphisms located in exonic regions, it is possible that those located in nonexpressed regions could affect the protein function through enhanced mutability because of altered DNA sequence context, increased RNA splicing events, altered transcript stability or translation, or interfered gene expression (35). A possible explanation for the worse outcome in patients homozygous for the G allele in our analysis might be the increased affinity and adhesiveness of E-selectin for its ligands in tumor cells in this subgroup. In addition, the ancestral allele may increase the release of E-selectin from the vascular endothelium, providing a soluble stimulant of

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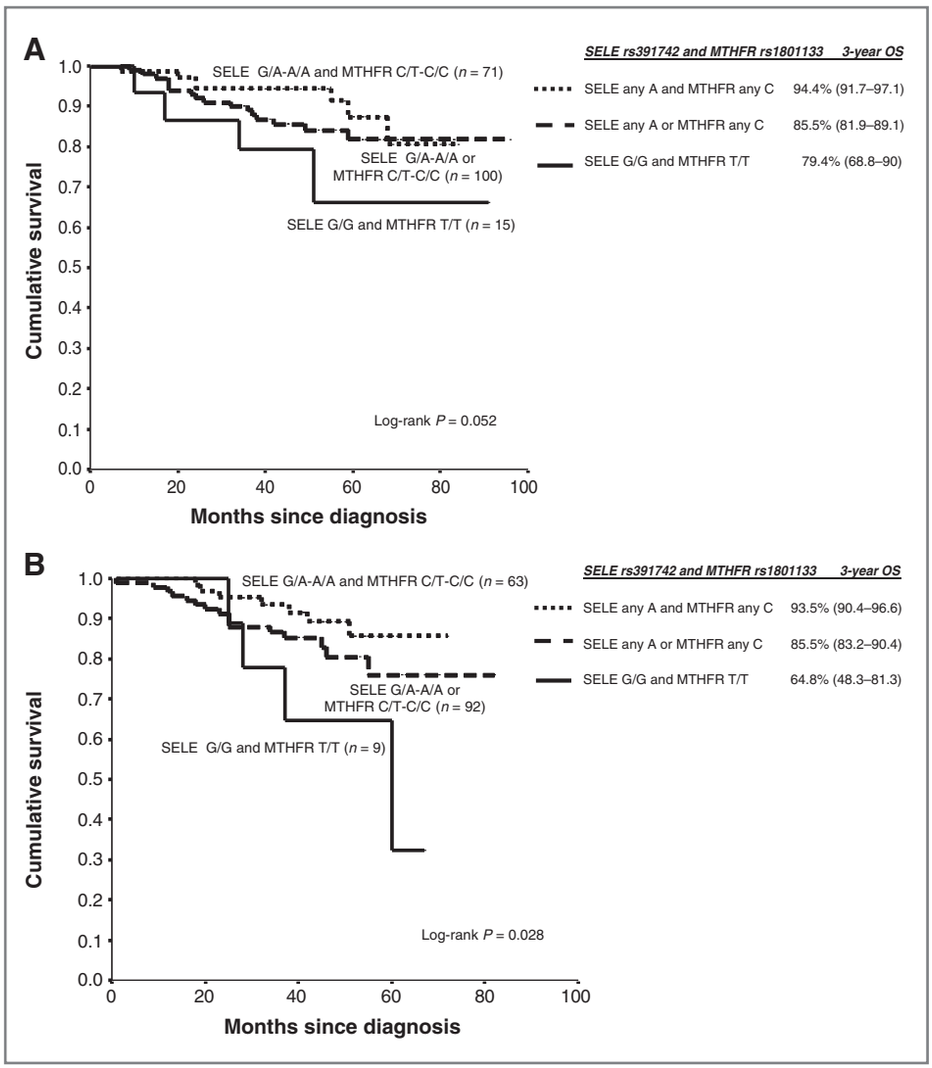

Figure 3. OS by combination of *SELE* rs3917412 and *MTHFR* rs1801133 SNPs in the training (A) and validation cohorts (B).

motility to direct cancer cells to the target organ, thus leading to a higher risk of metastatic seeding.

MTHFR is a key enzyme in the regulation of the intracellular folate levels, which mediates the irreversible conversion of 5,10-methylentetrahydrofolate (CH2FH4) to 5-methyltetrahidrofolate (CH3FH4). Experimental and clinical studies (36) have demonstrated that the balance of CH2FH4 and CH3FH4 is a crucial factor in fluoropyrimidines-induced cytotoxicity, as these drugs exert their effect by inhibiting TYMS through the formation of a ternary complex, involving 5-fluorouracil (5-FU), TYMS, and CH2FH4. Two common *MTHFR* SNPs, the rs1801133 677 C>T and the rs1801131 1298 A>C, are associated with decreased *MTHFR* enzymatic activity and thus increasing the intracellular levels of CH2FH4 available for the complex (36). Regarding to the *MTHFR* rs1801133 SNP, chemotherapy heterozygotes have a 65% lower enzymatic activity compared with wild-type colon cancer, whereas homozygous variants TT have a 30% lower activity. Furthermore, the TT genotype is associated with lower plasma folate, higher homocysteine levels, and lower DNA methylation levels *in vivo* (36). Therefore, it can be hypothesized that patients with the variant *MTHFR* alleles (677T or 1298C) will have increased sensitivity to fluoropyrimidine-based treatment than those bearing the common variants (677C or 1298A) and, possibly, an elevated risk of toxicity, by impairment of DNA synthesis and repair. Several studies, the majority conducted on patients with advanced colon cancer, have tried to clarify the possible predictive role of *MTHFR* SNPs on fluoropyrimidines activity and clinical outcome showing conflicting results (11–13, 26, 36–44). In our analysis, patients harboring the *MTHFR* rs1801133 T/T genotype were at higher risk to develop tumor recurrence. In accordance with our data, some studies show a negative effect on survival or response for the 677T allele (13, 37). However, other authors show no influence of *MTHFR* rs1801133 C>T genotype (12, 26, 38, 39) and others again show a positive effect of the T allele (11, 37, 40). Our results are not consistent with *in vitro* findings that suggest that patients

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with the T/T genotype would have reduced *MTHFR* activity compared with those with C/C and C/T genotypes, potentiating the antitumoral activity of fluoropyrimidines. Although the results from *in vitro* and clinical studies should not be directly compared, if we assume the functional effect of the *MTHFR* rs1801133 C>T allele, a possible explanation for our divergent findings may be that the cellular availability of 5,10-MTHF may depend not only on *MTHFR* genotype but also on other cofactors such as the folate dietary intake (45). Furthermore, population-based studies report an increased risk of malignancy and worse survival outcome in patients with the T/T genotype. It is possible therefore that the T/T genotype is an independent adverse prognostic factor regardless of fluoropyrimidine-based therapy (42).

In addition, we have shown that the combination of *SELE* rs3917412 and *MTHFR* rs1801133 SNPs could serve as a gender- and stage-specific genomic predictor of tumor recurrence in patients with stage II and III colon cancer. These results are consistent with previous reports which have found that polymorphisms in the *MTHFR* gene or other genes involved in relevant colon cancer pathways could predict gender- and stage-specific colon cancer outcome both in early-stage and advanced disease (44, 46–48). We have demonstrated that the *SELE* rs3917412 G/G and *MTHFR* rs1801133 T/T genotypes are significantly associated with a higher risk or recurrence in stage III patients, with a similar but nonsignificant trend in high-risk stage II patients. These data support the role of the *SELE* rs3917412 and *MTHFR* rs1801133 SNPs as molecular predictors of recurrence in both stages, but the limited sample size in the case of stage II disease makes it difficult to draw statistically significant conclusions. About the role of *MTHFR* SNPs as gender-specific prognostic markers, 2 previous reports have shown a significant association between the rs1801131 A1298C SNP and OS in female patients with metastatic colon cancer on 5-FU–based chemotherapy, but not in male patients (44, 46). However, in contrast to our findings, no differences in survival in men or women groups according to the *MTHFR* rs1801133 C677T SNP have been found. The underlying biologic mechanism of why sex may affect the association of *MTHFR* SNPs and clinical outcome is not clear. It has been postulated that female patients' estrogen status may interact with *MTHFR* genotype through decreasing homocysteine levels, but this impact is diminished in male patients (49). A second possible explanation is that sex is a strong predictor of methylation levels in the *MTHFR* gene, with males showing higher relative methylation levels (50).

Discrepancy between published data and our results could be explained by a number of factors, including differences in chemotherapy regimens, limited samples sizes, different technological platforms and sources of clinical material, and the absence of a validation cohort in most reports. Differences in ethnicity between populations may translate into contrasting pharmacogenomic interactions or activation of alternate pathways that affect tumor biology and behavior. Furthermore, it should be considered that the clinical significance of genotyping a single polymorphism is limited and may be inadequate to predict outcome in the adjuvant setting. Variations in genetic combinations and possible interdependence with other predictive enzymes are related to outcome and could also explain some of the difficulties in obtaining reproducible and uniform results when using single enzyme polymorphisms as predictive markers (13). Therefore, an approach that combines relevant SNPs of the genes involved in chemotherapy metabolism will yield a better, more logical model for explaining individual variations in treatment efficacy than single SNPs, as suggested in our study. This aspect is even more relevant if the pharmacogenetic analysis is performed in patients receiving combination chemotherapy with 2 or more drugs targeting independent biologic pathways (i.e., FOLFOX or CAPOX in our analysis).

Our study has a number of limitations such as its retrospective nature, although this point does not seem to affect the assessment of the primary endpoint (DFS). In addition, we carried out SNP testing in archival paraffin-embedded tumor samples. This could mean that we were unable to correct the effects of loss of heterozygosity (LOH), which would produce fewer heterozygous individuals than expected. However, concordance was found in the 95% to 99% of patients when genotypes for each SNP in tumor samples and adjacent nontumor tissue were compared. In addition, the fact that our population genotype distribution was in HWE with genotype frequencies in accordance with previous reports (28) suggests that LOH is of minor importance in our cohort. Finally, we have used a candidate polymorphism approach, which allowed us to focus on potentially functional SNPs reported in the literature but did not comprehensively cover all SNPs in the entire genome. Some important SNPs may have been missed or the observed associations may result from genetic linkages with other untyped SNPs. Nonetheless, despite its limitations, our analysis includes one of the largest populations studied with a long postoperative follow-up. Moreover, an almost unique advantage is that all patients were treated with oxaliplatin-based regimens along the same guidelines, presenting an exceptional opportunity for determining the effect of polymorphisms on oxaliplatin and fluoropyrimidines effect, avoiding bias introduced by varying treatment regimens and effects of prior therapies. Although imbalances in tumor grade, perineural invasion, bowel perforation, or adjuvant chemotherapy regimens between cohorts have been found, there are not statistically significant differences in the distribution of relevant SNPs genotypes according to these parameters neither in the training nor in the validation series. About this point, it should be emphasized that the ability to predict tumor recurrence and survival of our pharmacogenetic approach in 2 independent cohorts, despite some differences in baseline characteristics and adjuvant regimens, would reinforce the validity of our findings and the possibility to extrapolate them to populations other than the ones here studied.

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In conclusion, our results highlight new ways to explore in order to improve the prognostic stratification of early-stage colon cancer. Screening for *SELE* or *MTHFR* SNPs could effectively classify patients treated with oxaliplatin-based adjuvant chemotherapy into groups at low and high risk of disease recurrence, thereby adding prognostic value to significant clinicopathological risk factors used to assess these patients' prognosis. Therefore, new treatment options and more intensive strategies of follow-up may be explored after surgery in patients carrying these sequence variants. Although our findings require confirmation in large-scale prospective trials, they reinforce the concept that a pharmacogenetic approach potentially offers clinical value in directing personalized selection of adjuvant chemotherapy in patients with colon cancer in order to optimize clinical outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Custodio, J. Moreno-Rubio, A. Sánchez, J. Feliu
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Custodio, J. Moreno-Rubio, J. Aparicio, J. Gallego-Plazas, R. Yaya, J. Maurel, E. Burgos, A. Calatrava, E. Andrada, E. Díaz-López, A. Sánchez, J. Feliu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Custodio, J. Moreno-Rubio, E. Burgos, A. Sánchez, R. Madero, J. Feliu
Writing, review, and/or revision of the manuscript: A. Custodio, J. Moreno-Rubio, J. Aparicio, R. Yaya, J. Maurel, N. Rodríguez-Salas, A. Sánchez, J. Feliu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Custodio, J. Moreno-Rubio, A. Sánchez
Study supervision: A. Custodio, J. Moreno-Rubio, E. Burgos, A. Sánchez, J. Feliu
Other (histopathological evaluation of specimens, determination of histological subtypes, and determination of tumor stage): D. Ramos

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Molecular Cancer Therapeutics

Pharmacogenetic Predictors of Outcome in Patients with Stage II and III Colon Cancer Treated with Oxaliplatin and Fluoropyrimidine-Based Adjuvant Chemotherapy

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