

Role of primary miRNA polymorphic variants in metastatic colon cancer patients treated with 5-fluorouracil and irinotecan

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MicroRNAs are non-coding RNAs that can block mRNA translation and influence mRNA stability. Recent evidence indicates that miRNA variations can affect drug resistance, efficacy, and metabolism, opening new avenues of pharmacogenomics research. We investigated associations between polymorphisms in both miRNA-containing genomic regions (primary and precursor miRNA) and in genes related to miRNA biogenesis with clinical outcome in metastatic colorectal cancer (mCRC) patients treated with 5-fluorouracil and irinotecan (CPT-11). Eighteen single-nucleotide polymorphisms (SNPs) were analyzed in 61 patients. A significant association with tumor response and time to progression (TTP) was found for SNP rs7372209 in pri-miR26a-1 ($P=0.041$ and $P=0.017$, respectively). The genotypes CC and CT were favorable when compared with the TT variant genotype. In addition, SNP rs1834306, located in the pri-miR-100 gene, significantly correlated with a longer TTP ($P=0.04$). In the miRNA-biogenesis pathway, a trend was identified between SNP rs11077 in the exportin-5 gene and disease control rate ($P=0.076$). This study is the first to suggest a relationship between treatment outcome and SNPs in the miRNA-biogenesis machinery, in both primary and precursor miRNAs. Our results suggest that miRNA polymorphic variants might be useful predictors of clinical outcome in mCRC patients treated with 5-fluorouracil and CPT-11 combination. *The Pharmacogenomics Journal* (2011) 11, 429–436; doi:10.1038/tpj.2010.58; published online 29 June 2010

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Introduction

Colorectal cancer is the third most common cancer and the second leading cause of cancer death in the Western world.¹ Five-year mortality rates have modestly declined over the last three decades; however, the need remains for new predictive and prognostic biomarkers that can improve the ability of clinicians to determine the most effective treatment. This approach would allow to identify populations most likely to benefit from a specific type of therapy and a necessary step toward a tailored-made therapy. Pharmacogenomic studies emphasize on the use of genomic information to enhance the success of a given therapy, but recent evidence also indicates that mechanisms involving regulation of gene expression influence drug response.

MicroRNAs are non-coding, evolutionarily conserved RNAs that, through the regulation of gene expression at the post-transcriptional level, have an important role in multiple cellular processes. Several findings suggest that miRNAs affect chemosensitivity and drug resistance in cancer.^{2–4} Meng *et al.*² found that miR-21 and miR-200b inhibition increased the sensitivity of cholangiocarcinoma cell lines to gemcitabine by phosphatase and tensin homolog gene regulation. In addition, our group reported that the overexpression of miR-451 increases radiosensitivity in gastric cancer cells.³ More recently, it was shown, that at the translational level, miR-192 regulates the dihydrofolate reductase gene, an important target for anticancer drugs such as methotrexate, with the subsequent increase in methotrexate cell sensitivity *in vitro*.⁴

In addition, recent studies have reported that sequence variations in miRNA regions and miRNA-processing pathways may affect expression levels of mature miRNAs.^{5,6} Although single-nucleotide polymorphisms (SNPs) in miRNA regions are rare and considered unlikely to be functionally important,⁷ nucleotide variations within the seed sequence of the miRNA or within the sequence on primary (pri) and precursor (pre) miRNAs might affect miRNA processing and ultimately lead to modification of miRNA expression.⁸ Therefore, it is plausible that SNPs in miRNA-biogenesis machinery genes and miRNA-containing genomic regions may have a role in cancer development and treatment response. In this sense, the concept of miRNAs SNPs as a source of genetic variability that can influence the way that patients activate and metabolize drugs is emerging.^{9,10} The pharmacogenomic analysis of miRNAs may represent a novel and promising field of research that holds new possibilities for predicting drug behavior and improving drug efficiency.

Altered miRNA expression also has been strongly linked to cancer.^{11–14} Several factors have been implicated in miRNA abnormal expression in tumors, including gene amplification, deletion, epigenetic alterations, and abnormalities in miRNA processing and single-nucleotide substitution resulting in a mutation or SNP.^{15–17} An miRNA is a 22 nucleotides (nt) in length RNA that is transcribed as a pri-miRNA by RNA polymerase II.¹⁸ The pri-miRNA is cropped to release a hairpin-shaped precursor (pre-miRNA) of about 65 nt in length. Droscha (an RNase III) and its cofactor, DGCR8 (known as Pasha in *Drosophila melanogaster*), have an essential role in pri-miRNA processing.^{19,20} After this initial step, the nuclear transport factor exportin-5 (XPO5) exports the resulting pre-miRNAs into the cytoplasm by a Ran (a ras-related nuclear protein) GTP-dependent mechanism.²¹ In the cytoplasm, Dicer, a RNase III type protein associated with the transactivation response element RNA-binding protein (TRBP),²² recognizes the double-stranded (ds)RNA stem and releases a 21-bp dsRNA, from which the RNA-induced silencing complex will select one strand as the mature miRNA.²³ This complex also includes GEMIN3 and GEMIN4 and contributes to both miRNA processing and target gene silencing.^{24,25} Finally, the mature miRNAs guide RNA-induced silencing complex to mRNAs that

contain the miRNA target sequences²⁶ and negatively affect the expression levels of these mRNAs.²⁷

Several potential pathways could be under SNP influence. SNPs in miRNA-biogenesis genes might affect the expression of mature miRNAs and consequently miRNA-mediated regulation within the cell. On the other hand, SNPs in miRNA-containing genomic regions might affect the biogenesis of mature miRNAs and thus their expression within the cell. Furthermore, because small variations at the level of miRNAs could affect thousands of target mRNAs, SNPs in miRNA-related genes may significantly influence the proteome diversity of cells. Finally, SNPs in miRNA might represent ideal candidate biomarkers for cancer prognosis and prediction of clinical outcome. Thus, it would be of great interest to explore the association of polymorphic variations in miRNA-related genes and cancer therapies efficacy.

To investigate whether polymorphisms in miRNA-biogenesis genes and in miRNA-containing genomic regions may influence the outcome of metastatic colorectal cancer (mCRC) patients treated with 5-fluorouracil (5-FU) and irinotecan, we analyzed DNA obtained from 61 patients with mCRC treated with this combination. We focused on 18 SNPs, 11 of them in genes related to miRNA biogenesis (Droscha, DGCR8, XPO5, Ran, GEMIN3, GEMIN4, and TRBP) and the remaining from precursors (miR-146-a, miR-196a-2, miR-423, and miR-604) and primary miRNAs (miR-26a-1, miR-100, and miR-219-1). We hypothesized that these SNPs may affect miRNA expression, modify the proteome network, and have an impact on patient's clinical outcome.

Materials and methods

Study population, treatments, and clinical variables

Sixty-one Caucasian patients were retrospectively recruited from the Medical Oncology Service of the University Hospital Reina Sofía, Córdoba (Spain). Table 1a shows the clinical characteristics of the patient population at the time of their diagnosis. When we began the study (baseline), all patients had unresectable mCRC. Table 1b summarizes the characteristics of the patient population at baseline. They received a first-line chemotherapy with 5-FU (2250 mg m⁻² as a 48-h continuous infusion) and irinotecan (CPT-11, 180 mg m⁻²) on day 1, every 14 days. Tumor response was evaluated every 12 weeks using computed tomography scans. Response was defined according to Response Evaluation Criteria in Solid Tumors criteria as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD).²⁸ An evaluation of disease status was conducted. In the absence of disease progression, either clinical or radiological, therapy was continued until unacceptable toxicity or disease progression. At baseline, all patients showed a performance status (Eastern Cooperative Oncology Group) of 0–1. Thirteen patients (21%) had previously undergone surgical resection of the metastasis, but at baseline, all patients involved in the study had metastatic and unresectable disease that was at least C1 stage using the

Table 1 Characteristics of the study population

	n	%	ORR P-value*	TTP P-value [†]	OS P-value [‡]
<i>(a) Clinico-pathological characteristics at the time of diagnosis</i>					
<i>Age, years</i>					
Median	63		0.385	0.143	0.219
Range	41–77		0.658	0.576	0.354
≤65	31	51			
>65	30	49			
<i>Sex</i>					
Male	45	74	0.793	0.163	0.578
Female	16	26			
<i>Primary tumor site</i>					
Colon	32	52	0.878	0.866	0.684
Rectum	29	48			
<i>Differentiation grade</i>					
G1	8	14	0.551	0.19	0.586
G2	43	70			
G3	5	8			
Gx	5	8			
<i>Tumoral size</i>					
T2	3	5	0.638	0.882	0.988
T3	37	61			
T4	17	28			
Tx	4	6			
<i>Ganglion affection</i>					
N0	11	18	0.04	0.01	0.095
N1	24	39			
N2	7	12			
Nx	19	31			
<i>Stage on diagnosis</i>					
II	14	23	0.06	0.482	0.799
III	20	33			
IV	27	44			
<i>(b) Clinical characteristics at baseline</i>					
<i>Metastatic sites</i>					
Liver	36	59	0.058	0.6	0.566
Lung	20	33	0.508	0.948	0.959
Liver and lung	6	10	0.23	0.498	0.459
Other	19	31	0.009	0.851	0.459
<i>Organs with metastasis</i>					
1	44	72	0.138	0.644	0.221
≥2	17	28			
<i>Prior therapy</i>					
Tumor resection	57	76	0.5105	0.8847	0.0143
Metastasis resection	13	21	0.149	0.998	0.399
Adjuvant chemotherapy	31	51	0.004	0.159	0.877
Radiotherapy	11	18	0.892	0.659	0.664
Neoadjuvant chemotherapy	2	3	—	—	—
Living/deceased	35/26				

Abbreviations: ORR, overall response rate; OS, overall survival; TTP, time to progression.

*P-values were calculated as Pearson χ^2 or Mantel-Haenszel tests for categorical variables and Mann-Whitney or Kruskal-Wallis tests for continuous ones.

[†]P-value log-rank test.

second grid stage system.²⁹ Twenty-eight patients (46%) who had localized disease (stages II and III) at the time of their diagnosis and two patients (3%) who had undergone neoadjuvant chemotherapy before metastasis resection had previously received 5-FU, but none had previously been treated with CPT-11.

This study was approved by the institutional review board, and all patients provided written informed consent before recruitment.

Selection of genes and polymorphisms

We selected 11 SNPs in miRNA-biogenesis genes on the basis of the following criteria: (1) the SNP should reside in a functional region including exons, promoters, or untranslated regions (UTRs); (2) SNPs should have a reported minor allelic frequency >0.20 in a Caucasian population, as recorded in the SNP database (dbSNP: available to the public online at: <http://www.ncbi.nlm.nih.gov/SNP>).³⁰ In the case of multiple potentially functional SNPs within the same haplotype block (defined by the linkage coefficient $r^2 > 0.8$), only one SNP that fulfilled our criteria, was included.

To identify SNPs located in pri- and pre-miRNA genes, its precursor sequence was obtained from the microRNA registry (<http://microrna.sanger.ac.uk/>) and the corresponding genome regions for each miRNA gene by Blast analysis (<http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.htm>). The genomic region corresponding to each precursor miRNA was considered, including at least 100 base pair at the 5' and 3' ends, which covers the primary precursor of miRNA. Finally, the SNPs were found searching in the SNP database and selected when the minor allelic frequency was >0.20. The reported polymorphisms have been previously addressed in two case-control studies in renal³¹ and bladder cancer³², but at the time of writing, no data were available regarding the role of SNPs from microRNA-biogenesis genes and SNPs in miRNA-containing genomic regions in relation to treatment outcome. Table 2 summarizes the genes, nucleotide substitutions, position, amino acid changes, reference SNP identification numbers, and observed allele frequencies. Eleven SNPs were in seven genes of miRNA-biogenesis pathway (Drosha, DGCR8, XPO5, Ran, GEMIN3, GEMIN4, and TRBP), four SNPs were in pre-miRNAs (miR-146-a, miR-196a-2, miR-423, and miR-604), and three SNPs were in pri-miRNAs (miR-26a-1, miR-100, and miR-219-1).

Genotyping

Genomic DNA was isolated from peripheral blood using a QIAamp DNA extraction kit (Qiagen, Barcelona, Spain) according to the manufacturer's protocol. Genotyping was performed using TaqMan SNP Genotyping Assays (Applied Biosystems, Madrid, Spain) (Table 2). Allelic discrimination was carried out using the Applied Biosystems 7300 Real-Time PCR System. Duplicate samples were genotyped as a quality control.

Statistical analysis

Patient characteristics were reported as frequencies and percentages or as a median and interquartile range when

Table 2 SNPs examined and genotype frequencies

Gene name	SNP rs number	Position	Nucleotide change (M>m)	Amino acid change	No. of patients			Minor allele frequency ^a		Applied biosystem assay ID
					MM	Mm	mm	Observed	Reported	
Drosha	rs10719	3'UTR	C>T		39	20	2	0.20	0.23	C__7761648_10
DGCR8	rs3757	3'UTR	G>A		35	18	8	0.28	0.27	C__2539471_1_
	rs1640299	3'UTR	T>G		15	26	20	0.54	0.47	C__7543549_20
XPO5	rs11077	3'UTR	A>C		29	22	10	0.34	0.40	C__3109165_1_
RAN	rs14035	3'UTR	C>T		24	32	5	0.34	0.27	C__11351340_10
TRBP	rs784567	Promoter	C>T		10	34	17	0.56	0.48	C__9576934_20
GEMIN4	rs910924	Promoter	C>T		33	25	3	0.25	0.35	C__7470257_1_
	rs910925	exon 2	C>G	ALA>GLY	30	22	9	0.33	0.48	C__7470252_10
	rs4968104	exon 2	T>A	VAL>GLU	35	23	3	0.24	0.35	C__22274226_10
GEMIN3	rs197388	Promoter	T>A		39	19	3	0.20	0.29	C__32363724_30
	rs197412	exon 11	T>C	ILE>THR	21	33	7	0.39	0.47	C__923338_10
mir-146a	rs2910164	Pre-miRNA	G>C		36	22	3	0.23	0.24	C__15946974_10
mir-196a-2	rs11614913	Pre-miRNA	C>T		24	31	6	0.35	0.44	C__31185852_10
mir-423	rs6505162	Pre-miRNA	A>C		12	30	19	0.56	0.43	C__11613678_10
mir-604	rs2368392	Pre-miRNA	C>T		32	29	0	0.24	0.25	C__16218215_10
mir-26a-1	rs7372209	5UTR	C>T		26	29	6	0.34	0.29	C__29123986_10
mir-100	rs1834306	5UTR	C>T		21	31	9	0.40	0.44	C__11483095_10
mir-219-1	rs107822	5UTR	G>A		31	25	5	0.29	0.23	C__2215075_20

Abbreviations: mm, minor/minor allele; Mm, major/minor allele of the SNP; MM, major/major allele of the SNP; M>m, major>minor allele of the SNP; SNP rs number, single-nucleotide polymorphism reference identification number.

^aThe reported minor allele frequency was obtained from the dbSNP database.

appropriate. Groups were compared using the Pearson χ^2 or Mantel-Haenszel tests for categorical variables and Mann-Whitney or Kruskal-Wallis tests for continuous variables. The goodness-of-fit χ^2 test was adopted to assess the Hardy-Weinberg equilibrium.

Overall response rate (ORR) was determined by comparing patients showing CR or PR with patients showing SD or PD. In addition to response rate, the disease control rate (DCR), defined as an objective response (CR plus PR) plus SD, was determined and compared with patients showing disease progression. Response to therapy probability was modeled using univariate and multivariate logistic regression. *P*-values refer to the statistical significance of the coefficients. The same approach was followed to model the disease control probability, determined by comparing patients showing CR or PR or SD with patients experiencing PD.

Time to progression (TTP) was calculated from the date of first-line chemotherapy start to the date of first documented progression. Overall survival (OS) was calculated from the date of metastatic colon cancer diagnosis to the date of death. Probabilities of TTP and OS were calculated using the Kaplan-Meier method. Separate progression-free curves were estimated with Kaplan-Meier for SNP genotypes, and the log-rank test was used to assess statistically significant differences among curves. Hazard ratios with their 95% confidence intervals (95% CIs) were estimated using the univariate and multivariate Cox proportional hazards model in both TTP and OS analyses.

In the multivariate analysis, the association of each SNP with ORR, DCR, TTP, and OS was tested, and the model was

adjusted for possible confounders as well as for SNP interactions. In univariate analyses, *P*-values of all the 18 SNPs examined were corrected for multiple comparisons using permutation resampling techniques.³³ The *P*-values shown in the paper as *P*^{*} are the corrected ones.

Dominant, recessive, and additive genetic models were considered and tested for each SNP. All analyses were performed using SAS release 9.1.

Results

SNP frequencies

As shown in Table 2, genotype frequencies in the patient population were not significantly different from the minor allelic frequency values reported in the SNP database. All of the SNPs have minor allelic frequency values between 0.20 and 0.56. With the exception of two SNPs, rs3757 and rs2368392, we identified no evidence of departure from Hardy-Weinberg equilibrium.

Association of genotypes with tumor response

On an intent-to-treat analysis, the ORR was 41%, with 8 (13%) CR and 17 (28%) PR. In addition, 29 patients (48%) achieved SD, whereas PD were documented in 7 patients (11%). We observed a response rate (CR or PR) in 41% of the treated patients.

By univariate analysis, lymph node status and adjuvant chemotherapy were significantly associated with ORR. At baseline, we found a significant association of ORR with other metastatic sites such as bone or brain.

Table 3 Relevant associations of SNP rs7372209 in pri-miR-26a-1 with clinical outcome

Genotype	ORR					TTP						
	CR/PR n (%)	SD/PD n (%)	P-value*	OR ^a	95% CI	P-value ^a	MST (months)	95% CI	P-value*	HR ^b	95% CI	P-value ^b
<i>miR-26a-1</i> rs7372209												
CC/CT	23 (37)	32 (54)		1			18.32	13.37–24.09		1		
TT	2 (3)	4 (6)	0.034	0.1	0.01–0.99	0.044	9.18	3.96–18.52	0.017**	2.44	1.29–4.61	0.0035

Abbreviations: CI, confidence interval; CR, complete response; HR, hazard ratio; MST, median survival time; OR, odds ratio; ORR, overall response rate; PD, progression disease; PR, partial response; SD, stable disease; TTP, time to progression.

^aOR was adjusted for clinical factors. *P*-value from logistic regression model.

^bHR was adjusted for clinical factors. *P*-value from Cox regression model.

**P*-values were adjusted by multiple comparisons with permutation test.

***P*-value log-rank test.

By univariate analysis, rs7372209 SNP, located in the 5' region of the pri-miR-26a-1 gene, was significantly associated with ORR ($P^* = 0.041$). The effect of this SNP on response rate was stronger when homozygote wild type (CC) combined with heterozygote (CT) was compared with variant homozygote (TT) ($P^* = 0.034$). In multivariate analysis, performed by adjusting for clinical factors, this association remained statistically significant ($P = 0.044$) (Table 3). A trend toward an association with DCR was found for SNP rs11077 in the XPO5 gene when AA and AC genotypes were compared with the polymorphic variant CC ($P^* = 0.076$ and $P = 0.084$ in univariate and multivariate logistic analyses, respectively) (Supplementary Table 1).

Association of genotypes with TTP

At the time of the final analysis, the median follow-up time was 22.2 months (range, 17.31–31.18 months). A significant association was found between rs7372209 SNP in pri-miR-26a-1 and TTP ($P = 0.01$ log-rank test). Homozygote wild type (CC) and heterozygote (CT) showed twofold longer median TTP when compared with the homozygote variant (TT) (Figure 1). Patients carrying a C-containing genotype in miR-26a-1 SNP had a median TTP of 18.32 months (95% CI, 13.37–24.09), whereas median TTP was 9.18 months (95% CI, 3.96–18.52) in patients with polymorphic genotype TT variant, in both univariate ($P^* = 0.0017$) and multivariate ($P = 0.0035$) Cox proportional hazards regression analysis (Table 3).

Among the other SNPs analyzed, an association was observed between TTP and SNP rs1834306 located in the 5'UTR position of the pri-miR-100 gene ($P^* = 0.04$ log-rank test), although the adjusted Cox analysis showed only a trend toward an association ($P = 0.056$) (Supplementary Table 2). Median TTP for patients with the CC and CT genotypes was 18.52 months (95% CI, 13.37–23.47) compared with 12.62 months (95% CI, 7.70–16.75) for patients with the TT polymorphic genotype (Figure 2).

No further associations were found for the remaining miRNA-related genes. We also found a lack of interactions of the unfavorable genotypes of the two SNPs (in miR-26a-1 and miR-100 genes). In addition, none of the SNPs showed

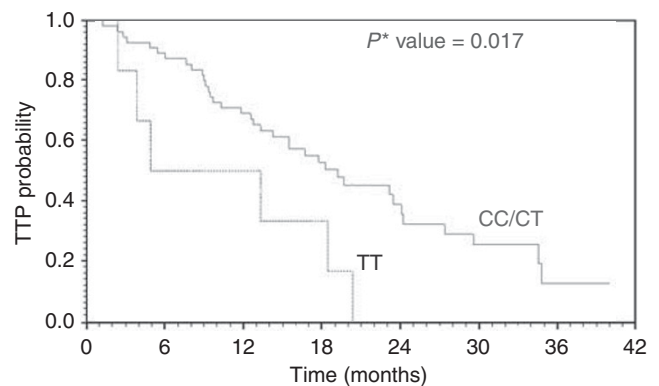


Figure 1 Kaplan–Meier method was used to assess the effect of single-nucleotide polymorphism (SNP) C>T rs7372209 in pri-miR-26a-1 on time to progression (TTP) in 61 patients. *P*-value was calculated using the log-rank test.

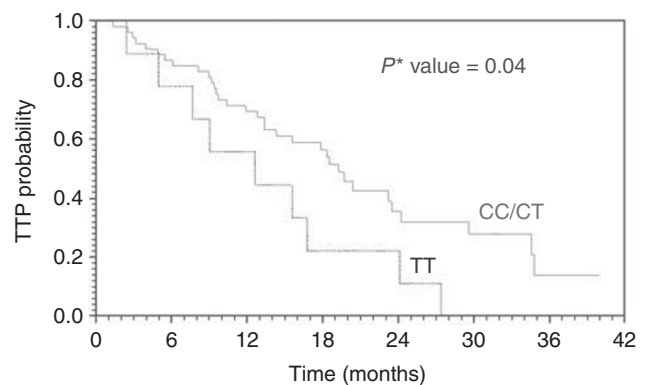


Figure 2 Kaplan–Meier method was used to assess the effect of single-nucleotide polymorphism (SNP) C>T rs1834306 in pri-miR-100 gene on time to progression (TTP) in 61 patients. *P*-value was calculated using the log-rank test.

a statistically significant association with OS. As noted, primary tumor resection was associated with an OS benefit in univariate but not in multivariate analysis.

Table 4 Putative target genes for miR-26a-1 and miR-100 predicted by TargetScan Human and miRANDA programs

Putative target genes	References
<i>miR-26a-1</i>	
SMAD1 (SMAD family member 1)	
TET1 (tet oncogene 1)	
E2F7 (E2F transcription factor 7)	
RB1 (retinoblastoma 1)	
CCND2 (cyclin D2)	Kota <i>et al.</i> ⁴⁰
CCNE (cyclin E)	
IGF1 (insulin-like growth factor 1)	
EZH2 (enhancer of zeste homolog 2, <i>Drosophila</i>)	Sander <i>et al.</i> ³⁹
PTEN (phosphatase and tensin homolog)	Huse <i>et al.</i> ⁴¹
<i>miR-100</i>	
CYP26B1 (cytochrome P450, family 26, subfamily B, polypeptide 1)	
HOXA1 (homeobox A1)	
PLK1 (Polo-like kinase 1)	Shi <i>et al.</i> ⁴⁵

TargetScan Human (version 5.1) and miRANDA programs are available online at: <http://www.targetscan.org/> and <http://www.microrna.org/>, respectively.

In silico analysis of miRNA putative targets

To explore the implications of our identified SNPs in translational regulation, we used the TargetScan Human (version 5.1)³⁴ and miRANDA³⁵ programs. We identified a list of putative genes targeted by miR-26a-1 and miR-100 (summarized in Table 4). Moreover, we performed a PubMed search with the two miRNAs as the keywords, selecting articles in which putative target genes for these miRNAs were previously functionally validated. These references are given in Table 4.

Discussion

The identification of molecular markers that can predict treatment response is one of the most important aims in pharmacogenomics and cancer research. Currently, there is a growing interest in the study of the association between miRNA expression in tumors and chemo- and radiosensitivity, both with regards to predicting or modulating sensitivity. Indeed, different miRNAs have been found to predict sensitivity to anticancer treatment; for example, miR-30c, miR-130a, and miR-335 are downregulated in various chemoresistant cell lines, and hsa-Let-7g and hsa-miR-181b are strongly associated with response to the 5-FU-based antimetabolite S-1.³⁶ These findings provide evidence that the variability in expression levels of miRNAs within the cell may influence chemotherapy sensitivity.

Polymorphisms present in miRNA-processing machinery, in pri- and in pre-miRNAs, could potentially influence expression levels of mature miRNAs, broadly affecting miRNA function. At the post-transcriptional level, miRNAs

regulate specific genes involved in multiple pathways, including cell death, cell proliferation, and stress resistance. Moreover, miRNAs can influence drug response, targeting proteins involved in the metabolism and/or the mechanism of action of specific drugs. Thus, several authors have defined miRNA pharmacogenomics as the study of miRNAs and polymorphisms affecting miRNA function to predict drug behavior and to improve drug efficacy. In this context, we investigated the potential correlation between SNPs in miRNA-containing genomic regions and in miRNA-biogenesis machinery genes and the clinical outcome of mCRC patients treated with first-line 5-fluoruracil and CPT-11 combination.

To our knowledge, our data are the first to provide evidence that common SNPs in miRNA-related genes may have a role in predicting clinical outcome in mCRC patients treated with 5-FU and CPT-11. We found that SNPs in miRNA-containing genomic regions and in miRNA-biogenesis machinery genes may be useful predictors of 5-FU and CPT-11 efficacy in mCRC patients. The SNP rs7372209 in miR-26-a-1 was associated with both ORR and TTP. Allele C appears to be a favorable factor, as was confirmed comparing the median TTP of CC + CT genotypes to the homozygote variant genotype (TT). Furthermore, the SNP rs1834306 in the 5'UTR region of pri-miR-100 and the SNP rs11077 in the XPO5 gene were also found to be associated with TTP and DCR, respectively.

The molecular mechanism by which these polymorphisms lead to improving the efficacy of a drug is not yet understood. Indeed, for only a few miRNAs are the biological functions only beginning to be more clearly understood. In our opinion, these SNPs may affect miRNA primary transcription and then expression of the mature miRNA. Finally, the ectopic expression of mature miRNAs may affect the translational suppression of their targets and consequently influence drug response. Although it is not possible at this time to further define the mechanism through which these SNPs affect response to therapy, several studies have reported a correlation between miRNA expression and the effect on sensitivity to chemotherapy. For example, the sequence variations in miRNA genes, including pri-miRNAs, pre-miRNAs, and mature miRNAs, could potentially influence the processing and/or target selection of miRNAs.⁶ These findings support our initial hypothesis that some variant alleles in microRNA-containing genome regions and/or in miRNA-processing machinery, by changing the expression of mature miRNAs, might be associated with cancer response. However, the elucidation of the mechanisms underlying SNP function and overcoming their role in chemotherapy resistance warrants further insight.

Interestingly, when we performed the *in silico* analysis for putative target genes of these miRNAs, we found that some of them have been implicated in colon cancer tumorigenesis and clinical outcome. For example, E2F transcription factor 7 (E2F7) and SMAD family member 1 (*SMAD1*) genes were identified by TargetScan as putative targets of miR-26a-1. E2F transcription factors, such as E2F7, have an essential role in the regulation of cell-cycle progression,³⁷ and SMAD

proteins are members of the transforming growth factor- β pathway protein family, involved in regulation of cell proliferation, differentiation, and apoptosis.³⁸ Furthermore, recent studies have shown that the ectopic expression of miR-26a influences cell-cycle progression by targeting the bona fide oncogene enhancer of zeste homolog 2 (EZH2), a polycomb protein, and global regulator of gene expression³⁹; in addition, a recent work has shown that in liver cancer cells, ectopic expression of miR-26a induces cell-cycle arrest by direct targeting of cyclins D2 and E2.⁴⁰ Finally, Huse *et al.*⁴¹ reported that miR-26a is a direct regulator of the expression of phosphatase and tensin homolog, a tumor suppressor gene frequently altered in cancer.⁴²

Interestingly, the putative target genes of miR-100 predicted by the TargetScan Human program may be involved in colon cancer pathogenesis, as well. For instance, we found that one potential target of miR-100 is the gene HomeoboxA1 (*HOXA1*). Furthermore, in searching using the miRANDA program, as putative target gene, insulin-like growth factor 1 receptor (*IGF1R*) was found. This gene is highly overexpressed in most malignant tissues, where its protein functions as an anti-apoptotic agent by enhancing cell survival, and recent evidence suggests that *IGF1R* is a key player in cancer therapeutic resistance.⁴³ Recent reports have also shown that a decreased expression of miR-100 contributes to malignancy in oral cancer cells and that it is associated with loss of sensitivity to ionizing radiation.⁴⁴ For the validated target of miR-100, we found only the Polo-like kinase 1 (*Plk1*) gene, a key regulator of mitosis.⁴⁵ Furthermore, *Plk1* has been proposed as a new prognostic biomarker in colon cancer patients.⁴⁶ Finally, the SNP rs11077 in the XPO5 gene showed a trend toward an association with DCR. The exportin-5 protein is a pre-miRNA-specific export carrier involved in shuttling pre-miRNA into the cytoplasm through a saturable carrier-mediated process.⁴⁷ It is possible that SNPs in the 3'UTR region of XPO5 could be associated with altered expression levels of this protein and consequently with variations in mature miRNA expression in the cytoplasm.

Lastly, two SNPs, the rs3757 and the rs2368392, were found in Hardy-Weinberg disequilibrium in our population. We argued three possible explanations for these findings: first, it could be due to linkage disequilibrium with other near loci. Loci that are phenotypically neutral but are somehow associated with the disease-susceptibility locus, such as genetic markers in linkage disequilibrium with the disease-susceptibility locus, experience disproportionate genotype selection. Second, the small number of patients recruited in the study could explain these departures. Finally, these polymorphisms could be associated with colorectal cancer risk. Alleles within genotypes that confer greater susceptibilities are usually represented in the sample at disproportionately high rates. However, a case-control study would be necessary to investigate this last interesting hypothesis.

In conclusion, we investigated the influence of SNPs in miRNA-related genes on clinical outcome in mCRC patients homogeneously treated with 5-FU and CPT-11. Although

our results must be validated in larger prospective studies, they highlight a potential role of these SNPs as predictive biomarkers. Changes in precursor and primary sequences of miRNAs could have an important effect on post-transcriptional regulation, influencing miRNA expression levels and consequently the cellular proteomic network. The described putative and validated targets of miR-26 and miR-100 are known as important genes involved in colon cancer development, although they have not directly been related to 5-FU and/or irinotecan metabolism or mechanism of action. We may therefore speculate that our relevant SNPs may be associated with response to treatment but not with a response to a specific drug combination. Unfortunately, the design of our study precludes further conclusions. Future studies are necessary to explore the functional role of this new class of SNPs. Finally, in the future, these SNPs may be used as a decision-making tool to assist oncologists in selecting the most appropriate systemic therapy for each patient.

Conflict of interest

The authors declare no conflict of interest.

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