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Single nucleotide polymorphisms in the IGF-IRS pathway are associated with outcome in mCRC patients enrolled in the FIRE-3 trial

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The Insulin-like growth factor (IGF)/IGF-receptor pathway with its scaffolding proteins Insulin Receptor Substrate (IRS)1 and IRS2 are crucial regulators of metabolism and progression in metastatic colorectal cancer (mCRC). The goal of the study was the identification of predictive and prognostic markers among *IRS1*, *IRS2*, *IGF*1 and *IGF-1R* SNPs in mCRC patients enrolled in the FIRE-3 trial. Four SNPs of *IRS* (*IRS1* rs1801278, rs1801123; *IRS2* rs1805097, rs2289046) and four SNPs of *IGF1-IGFR1* (rs6214, rs6220, rs2946834, rs2016347) were analyzed by PCR/direct-sequencing in the FIRE-3 trial. The relation of SNPs with PFS and OS was evaluated through Kaplan–Meier method and log-rank test in the overall population and in subgroup according to *RAS* status and treatment arm. In the overall population *IRS1* rs1801123 C/- carriers (*N*= 105) achieved significantly worse OS compared to T/T (*N* = 464) in univariate (HR = 1.32 [95%CI 1.03-1.70], $p = 0.029$) and in multivariable. Similar results were observed among *RAS* wild type. Patients with *IGF1* rs2946834 T/- variant (*N*= 280) achieved improved PFS compared to C/C (*N* = 257) in univariate (HR = 0.77 [95%CI 0.64-0.92], $p = 0.004$) and in multivariable. In the *RAS* wild-type subgroup *IGF1* rs2946834 T/- carriers showed better PFS and OS compared to C/C (univariate HR for PFS = 0.65 [95%CI 0.51–0.81], $p < 0.001$; multivariable HR for PFS = 0.63 [95%CI 0.50–0.81], $p < 0.001$). IRS1 rs1801123 SNP was identified as a new prognostic marker for mCRC. *IGF1* rs2946834 was confirmed as prognostic factor in the overall population and in *RAS* wild type patients. Our findings underline the importance of IGF downstream signaling pathway in *RAS* wild-type mCRC patient.

The Insulin Growth Factor-1 (*IGF*)-/Insulin-like Growth Factor- Receptor1 (*IGF-1R*) pathway is a key mediator of the response of tumor cells to the metabolic microenvironment and the regulation of tumor cell metabolism, proliferation, survival and angiogenesis.[1–3] This signaling pathway has been identified as a critical determinant of colorectal cancer (CRC) development,[2,3] and many evidences showed hyperinsulinemia as determinant of CRC risk as well as possible prognostic factor.[4–7]

Insulin receptor substrate (*IRS*) 1 and *IRS2* are scaffolding proteins regulating downstream signaling of *IGF/IGF-1R*. They activate and regulate phosphoinositide 3-kinase (*PI3K*) and extracellular signal regulated kinase (*ERK*) pathways that are implicated in metabolism and protein synthesis, cell growth as well as key regulators of colorectal cancer development and progression and tightly connected to the *EGFR* signaling pathway.[8,9] *IRS1* is overexpressed in CRC patients and has been associated with cancer progression and development of metastases. *IRS2* mRNA and protein expression positively correlate with normal colorectal epithelium transitioning to adenoma, and subsequently, to adenocarcinoma.[10,11] Amplification and mutations of *IRS2* were recently identified in tumors with increased sensitivity to anti-EGFR therapy.[12] *IRS1* and *IRS2* polymorphisms have also been independently associated with increased CRC risk. In particular carriers of *IRS1* rs1801278 T/- genotype showed higher incidence of

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What's new?
Newly identified genetic variants could predict survival in colorectal cancer. The insulin growth factor (IGF) and IGF-1R pathway mediate the growth and spread of tumor cells. These authors studied polymorphisms in *IGF*, *IGF-1R*, and two downstream scaffolding proteins, *IRS1* and *IRS2* to determine whether they could help predict outcomes. They confirmed previous findings that people carrying a particular SNP in *IGF1* have better survival; they also picked out a SNP in *IRS1* that predicts outcome in patients with wild-type RAS. These markers could help identify patients who are more likely to benefit from drugs that target the IGF-1R pathway.

CRC, while patients with *IRS2* rs1805097 C/T genotype had significantly reduced the risk of developing CRC.[13,14]

IGF-1R is frequently overexpressed in human colon cancer cells[15] and has been related to the promotion of growth and malignant transformation of adenomatous polyps[16] and to the development of distant metastases in murine models. The activation of insulin/*IGF*-dependent pathways has been identified as a possible mechanism of CRC resistance to conventional and targeted therapeutic agents and in particular to anti-EGFRs.[17,18] This observation is supported by the identification of a strong cross talk activity between *IGF-1R* and *EGFR* blockade and their downstream signaling pathways. When *IGF-1R* signaling is up regulated it might be able to overcome the inhibitory activity of anti-EGFRs leading to the activation of *RAS* and *PI3K*/*Akt* pathways and stimulating proliferation, cell growth and apoptosis inhibition.[9] Our group retrospectively investigated the association of six potentially functional *IGF1* and *IGF1R* polymorphisms with survival parameters in a cohort of 130 chemotherapy refractory mCRC patients enrolled in the IMC-0144, a phase II clinical trial of cetuximab monotherapy. In univariate and multivariable analyses five IGF-pathway SNPs were significantly associated with progression-free-survival (PFS) and/or overall survival (OS). The effect of the SNP in terms of outcome prediction was even more significant in the subgroup of patients with *KRAS* wild type tumor[19] (Supporting Information Fig. 1 shows the *IGF-IGFR-IRS* pathway).

The present study aimed at (*i*) the identification of a prognostic/predictive role for selected *IRS1* and *IRS2* SNPs and (*ii*) the prospective validation of previous findings on *IGF-IGF-1R* SNPs in the randomized phase III FIRE-3 trial.[20]

Material and Methods

Patient population

A total of 614 tissue samples deriving from primary of patients treated with first-line FOLFIRI plus bevacizumab and FOLFIRI plus cetuximab in the randomized, open-label, phase III FIRE-3 trial[20] were available for the present analysis. Patients had histologically confirmed stage IV colorectal adenocarcinoma, measurable disease according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.0 and did not receive prior treatment for metastatic disease, nor exposure to irinotecan, cetuximab (Arm A), or bevacizumab (Arm B). Standard inclusion and exclusion criteria were applied. Patients received bevacizumab 5 mg/kg or cetuximab 400 mg/kg, followed by irinotecan 180 mg/m2, leucovorin 200 mg/m2, 5-fluorouracil 400 mg/m2 bolus infusion and 5-fluorouracil 2,400 mg/m2 as a 48-hr continuous infusion. Treatment was administered every 2 weeks until disease progression, intolerable toxicities, or patient withdrawal. Responses were measured by contrast-enhanced computed tomography (CT) scans after 6 and 12 weeks of treatment, and every 10 weeks thereafter according to RECIST v1.0.

SNP selection

IRS1 and *IRS2* polymorphisms were chosen for investigation according to the following criteria:

- minor allele frequency>5% in Caucasians according to the ENSEMBL database (http://www.ensembl.org/index.html)
- citation on Pubmed and possible relation with cancer
- functional or predicted functional relevance of gene transcription or protein expression. Functional significance was predicted based on information provided by the National Institute of Environmental Health Science SNP Function Prediction, Queen's University F-SNP, and the location of the SNP in the protein-coding region of the gene (http://snpinfo.niehs.nih.gov/snpinfo/snptag.htm).

IGF1 and *IGFR1* polymorphisms, following the above specified inclusion criteria, were further selected based on the results of our previous study if an association with either PFS or OS was maintained in the multivariable model.[19]

Supporting Information Table 1 shows genes, SNP identification numbers, location and adopted forward and reverse primers. Genotyping procedures are described in the supplementary informations.

Genotyping

Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues of patients using the QIAmp Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol (www.qiagen.com). PCR-based direct DNA sequence analysis using ABI 3100 A Capillary Genetic Analyzer and Sequencing Scanner v1.0 (Applied Biosystems, Waltham, MA) was performed for genotyping the SNPs. The extracted DNA was amplified using the primer sets shown in Supporting Information Table 1, and analyzed by PCR-based direct DNA sequencing. The investigator analyzed sequencing data using the ABI Sequencing Scanner v1.0 (Applied Biosystems, Life Technologies, Grand Island, NY) and was blinded to the clinical data set.

Statistical methods

The primary objective of the study was the exploratory analyses on the prognostic/predictive role of *IRS1* and *IRS2* SNPs in mCRC patients enrolled in the randomized phase III FIRE-3 trial. Subgroup analyses according to treatment arm, *KRAS* and extended *RAS* status were preplanned.

Secondary objective was the validation of previous findings on *IGF*1 and *IGFR1* SNP as potential predictive/prognostic molecular markers for cetuximab efficacy in mCRC patients. The above specified subgroup analyses were performed.

Primary and secondary clinical endpoints were OS and PFS, respectively. OS was defined as the time from treatment start to death due to any cause. PFS was defined as the time from treatment start to first documented disease progression or death due to any cause. Patients were censored at the time of last follow-up if no events occurred. Patients were defined as responders when achieving complete or partial response and non-responders when stable or progressive disease occurred as defined by RECIST 1.0 criteria. Differences between baseline characteristics of Arm A and Arm B patients were analyzed by means of χ^2 test for categorical factors and the Wilcoxon rank sums test for continuous variable.

Allelic distribution of polymorphisms was tested for deviation from Hardy–Weinberg equilibrium (HWE) using χ^2 test. Linkage disequilibrium among SNPs was assessed using *D*' and r^2 values, and the haplotype frequencies of the two genes were inferred using HaploView version 4.2 (http://www.broad.mit.edu/mpg/haploview). Kaplan–Meier curves, log-rank test and Cox proportional hazard models were applied to evaluate the association between SNPs with PFS and OS in univariate and multivariable model. The association between SNPs and the baseline characteristics was examined using χ^2 test. Baseline characteristics that remained significantly associated with PFS and OS in multivariable model after backward elimination procedure with $p < 0.1$ criteria were included in the final model. *p* values for all SNPs were adjusted for multiple testing using p_{act} method, a modified test introduced by Conneely and Boehnke that was applied for the correlated tests due to linkage disequilibrium and different modes of inheritance considered.[21] Fisher's exact test was used to evaluate the association between SNPs and tumor response. The true mode of inheritance of SNPs was not a priori established, and we assumed a codominant, dominant or recessive model whenever appropriate.

At the time of the current data analyses, we observed 391 deaths out of 600 evaluable patients. We would have 80% power to detect the effect of a SNP on OS with HRs ranged from 1.33 to 1.59 using two-sided 0.05-level log-rank test when the dominant model with the minor allele frequencies of 0.05 to 0.40 is considered.

SAS 9.4 (SAS Institute, Cary, NC) was used to perform all analyses. All tests were two-sided at a significance level of 0.05.

Results

600 out of 614 patients were evaluable for the present study: 305 patients were enrolled in arm A and 295 enrolled in arm B. Main patient's characteristics are described in Supporting Information Table 2: 93% of patients in arm B presented T stage >2 compared to those in arm A (89%; $p = 0.006$). No other relevant differences were observed according to treatment arm. Details regarding the number of successfully genotyped samples in evaluable patients for each SNP are described in Supporting Information Table 1.

Median follow-up, median PFS and median OS were 40.9, 9.9 and 24.8 months in the overall population; 41.8, 9.6 and 26.5 months in arm A; and 40.8, 10.1 and 24.2 months in arm B.

Association of patient baseline characteristics with PFS and OS in the overall population and the two arms were summarized in Supporting Information Tables 3–5. In the overall population, ECOG PS 1, right- sided primary tumor, *RAS* and *BRAF* mutations T stage> 2 were statistically associated with worse PFS and OS in the univariate model. An association with worse PFS was observed in females while an association with worse OS was identified in patients with liver metastases, number of metastatic sites >2, synchronous metastatic disease, and *KRAS* mutations. In respect to different treatment arms, the same factors were associated with PFS or OS. However, the effect of RAS mutations, RAS mutations and T stage was not significant in patients treated with FOLFIRI plus bevacizumab. All the genotyped SNPs were on HWE ($p > 0.05$) and the frequencies of the selected SNPs were in line with literature data. IRS2 rs2289046 and IRS2 rs1805097 were in linkage disequilibrium with *D*' of 0.72 and r^2 of 0.41. Other disequilibrium was not observed.

Primary objective results

In the overall population patients with *IRS1* rs1801123 C/- ($N = 105$) achieved a significantly worse OS compared to T/T variant carriers ($N = 464$) in the univariate analyses (median OS: 21.3 *vs.* 26.4 months, HR = 1.32 [95%CI 1.03–1.70], $p = 0.029$; Fig. 1*a*) and remained significant in the multivariable model adjusted for sex, ECOG performance status, primary tumor site, liver only disease, number of metastases, RAS status, BRAF status, T stage and stratified by treatment arm (HR = 1.28 [95%CI 1.00–1.66], $p = 0.054$; Table 1).

Similar results were observed among *KRAS* wild type patients ($N = 476$; median OS: C/- 21.3 *vs.* T/T 27.6 months; univariate analyses HR = 1.38 [95% CI 1.04–1.85], $p = 0.025$; multivariable model HR = 1.41 [95% CI 1.05–1.90], $p = 0.022$; Table 2) and among *RAS* wild type patients ($N = 379$; C/- 21.8 *vs.* T/T 28.6 months; univariate analyses HR = 1.39 [95%CI 1.01–1.90], $p = 0.042$; multivariable model HR = 1.48 [95%CI 1.07–2.05], $p = 0.019$; Table 3, Fig. 1*b*). In multiple testing including all SNPs, none of them remained significantly associated with OS. No differences were observed looking at patients

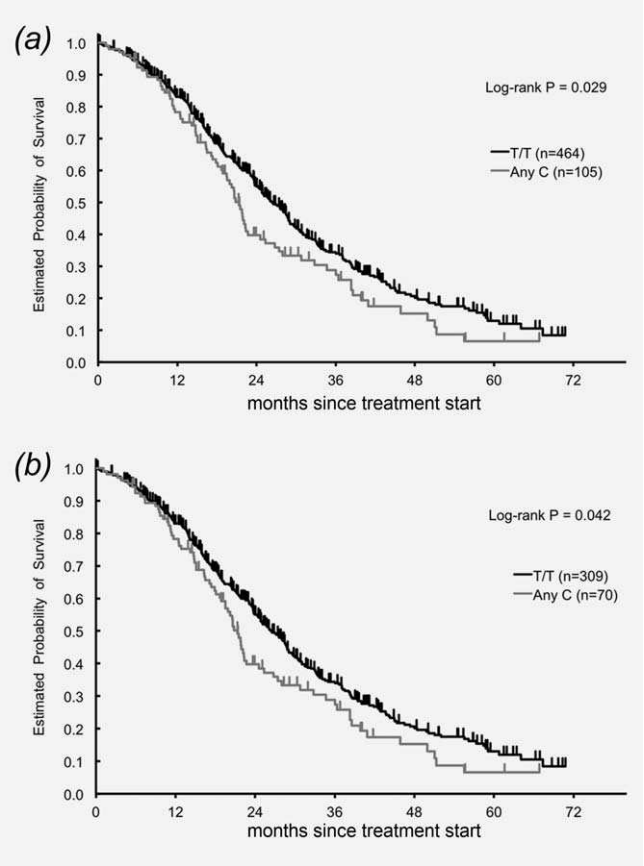

Figure 1. (*a*): IRS1 rs1801123 and OS in overall population of FIRE 3 trial. (*b*) IRS1 rs1801123 and OS in RAS wild type patients.

treated in arm A and arm B separately (Supporting Information Tables 6 and 7).

Secondary objective results

In the overall population patients with *IGF1* rs2946834 T/- ($N = 280$) achieved an improved PFS compared to C/C variant carriers ($N = 257$) in the univariate analyses (median PFS T/- 10.5 *vs*. C/C 9.7 months, HR = 0.77 [95%CI 0.64–0.92], $p = 0.004$; Fig. 2*a*) and in the multivariable model with an HR = 0.78 [95%CI 0.64–0.94], $p = 0.010$ (Table 1). Similar results were observed among *KRAS* wild type patients (Table 2). It remained significantly associated with PFS after multiple testing in both overall patients and KRAS wild type patients (adjusted $p = 0.074$ and 0.007, respectively).

In the *RAS* wild type subgroup patients with *IGF1* rs2946834 T/- retained their significantly improved PFS compared to C/C variant carriers at both univariate (HR = 0.65 [95%CI 0.51–0.81], $p < 0.001$; Fig. 2*b*) and multivariable analyses (HR = 0.63 [95%CI 0.50–0.81], $p < 0.001$). It remained significantly associated with PFS after multiple testing in *RAS* wild type patients (adjusted $p = 0.002$). Similar results were observed also in terms of OS both at univariate (HR = 0.75 [95%CI 0.58–0.99], $p = 0.037$), multivariate (HR = 0.65 [95%CI 0.49–0.87], $p = 0.003$) and after adjustment for multiple testing ($p = 0.024$). Moreover, patients with *IGF1 rs6220* G/- ($N = 170$) showed improved PFS compared to A/A carriers ($N = 198$; median PFS 11.3 *vs*. 9.9, univariate analyses HR = 0.77 [95%CI 0.62–0.97], $p = 0.024$; multivariable model HR = 0.78 [95% CI 0.61–0.98], $p = 0.034$; Table 3). However, it showed no significance after the multiple testing.

Among patients treated within arm A (FOLFIRI + cetuximab), *IGF1* rs2946834 T/- ($N = 126$) achieved a significantly improved PFS compared to C/C variant carriers ($N = 144$) in the univariate analyses (median PFS 10.4 *vs*. 9.5 months, HR = 0.76 [95%CI 0.59–0.99], $p = 0.037$). The association was lost in the multivariable model (HR = 0.80 [95% CI 0.60–1.05], $p = 0.11$). Interestingly in the subgroup of *KRAS* wild type, T/- carriers achieved better PFS compared to C/C (HR = 0.66 [95% CI 0.49–0.88], $p = 0.004$) and the association was maintained in the multivariable model (HR = 0.71 [95% CI 0.52–0.98], $p = 0.039$). Similar results were observed among *RAS* wild type patients, in this subgroup T/- carriers showed improved PFS and OS in the univariate (HR for PFS = 0.65 [95% CI 0.47–0.91], $p = 0.010$; HR for OS = 0.64 [95%CI 0.42–0.97] $p = 0.029$) and in the multivariable model (HR for PFS = 0.69 [95% CI 0.48–0.99], $p = 0.047$; HR for OS = 0.61 [95% CI 0.38–0.98] $p = 0.040$; Supporting Information Table 6).

Among patients treated within arm B (FOLFIRI + bevacizumab) carriers of *IGF1* rs2946834 T/- ($N = 154$) achieved a significantly improved PFS compared to C/C variant carriers ($N = 113$) in the univariate analyses (median PFS 10.5 *vs*. 9.9 months, HR = 0.77 [95% CI 0.59–1.00], $p = 0.045$) and the association was marginal in the multivariable model (HR = 0.77 [95% CI 0.58–1.03], $p = 0.074$). In the subgroup of *KRAS* wild type, T/- carriers achieved better PFS compared to C/C (HR = 0.71 [95% CI 0.53–0.95], $p = 0.016$) with significant results also in the multivariable model (HR = 0.71 [95% CI 0.52–0.97], $p = 0.031$). Similar results were observed among *RAS* wild type patients, in this subgroup T/- carriers showed improved PFS in the univariate (HR = 0.63 [95%CI 0.45–0.87], $p = 0.003$ and in the multivariable model (HR = 0.59 [95% CI 0.41–0.83], $p = 0.003$; Supporting Information Table 7).

Discussion

The present study identified *IRS1* rs1801123 C/T SNP as prognostic factor for metastatic CRC patients enrolled in the FIRE-3 trial irrespectively from treatment with bevacizumab or cetuximab. Interestingly, in subgroup analyses of *RAS* wild type patients the prognostic effect of *IRS1* rs1801123 C/T was confirmed and no effect was observed among patients carrying *RAS* mutations. Although an association of *IRS* SNP and CRC risk was already demonstrated, to the best of our knowledge this is the first report showing a prognostic role for *IRS1* SNP with particular regard to *RAS* wild type mCRC patients.

IRS1 rs1801123 C/T SNP has been associated with splicing and post-transcriptional regulation of IRS1 protein; *IRS*-1

Table 1. Association of SNPs and outcome in the overall population (Arm A + B)

	Tumor response				Progression-free survival					Overall survival				
	N	Yes	No	*p* value*	Median (95%CI), months	Univariate HR (95%CI) †	*p* value*	Multivariable HR (95%CI) ‡	*p* value*	Median (95%CI), months	Univariate HR (95%CI) †	*p* value*	Multivariable HR (95%CI) ‡	*p* value*
IRS1 rs1801278				0.41			0.31		0.088 (0.42)			0.87		0.96 (1.00)
C/C	495	279 (63%)	167 (37%)		9.7 (9.0, 10.2)	1 (Reference)		1 (Reference)		24.8 (22.7, 27.6)	1 (Reference)		1 (Reference)	
Any T	78	44 (69%)	20 (31%)		12.2 (9.9, 13.4)	0.87 (0.67, 1.14)		0.79 (0.61, 1.04)		25.1 (21.3, 36.0)	0.98 (0.72, 1.32)		0.99 (0.73, 1.35)	
IRS1 rs1801123				0.47			0.92		0.30 (0.51)			***0.029***		***0.054*** (0.27)
T/T	464	269 (65%)	147 (35%)		9.9 (9.2, 10.4)	1 (Reference)		1 (Reference)		26.4 (23.9, 28.8)	1 (Reference)		1 (Reference)	
Any C	105	55 (60%)	36 (40%)		10.1 (8.6, 11.8)	0.99 (0.79, 1.24)		0.88 (0.70, 1.12)		21.3 (18.7, 22.7)	1.32 (1.03, 1.70)		1.28 (1.00, 1.66)	
IRS2 rs2289046				0.78			0.60		0.21 (0.62)			0.55		0.27 (0.62)
T/T	256	145 (64%)	80 (36%)		10.0 (9.0, 10.9)	1 (Reference)		1 (Reference)		23.5 (21.0, 26.1)	1 (Reference)		1 (Reference)	
Any C	306	175 (63%)	102 (37%)		9.9 (9.2, 10.5)	0.95 (0.80, 1.14)		0.89 (0.74, 1.07)		26.5 (23.8, 28.7)	0.94 (0.77, 1.15)		0.89 (0.72, 1.10)	
IRS2 rs1805097				0.92			0.18		***0.045*** (0.26)			0.30		0.12 (0.46)
A/A	252	143 (65%)	78 (35%)		10.0 (8.9, 10.6)	1 (Reference)		1 (Reference)		23.1 (20.6, 25.2)	1 (Reference)		1 (Reference)	
Any G	293	168 (64%)	95 (36%)		9.9 (9.2, 10.7)	0.88 (0.74, 1.06)		0.83 (0.69, 1.00)		26.3 (23.4, 28.8)	0.90 (0.73, 1.10)		0.84 (0.68, 1.05)	
IGF1 rs6214				0.53			0.24		0.23 (0.55)			0.18		0.14 (0.46)
G/G	188	105 (63%)	62 (37%)		9.3 (8.5, 10.4)	1 (Reference)		1 (Reference)		25.4 (23.2, 29.8)	1 (Reference)		1 (Reference)	
Any A	273	163 (66%)	84 (34%)		10.5 (9.9, 12.2)	0.89 (0.73, 1.09)		0.88 (0.72, 1.08)		24.5 (21.3, 28.1)	1.17 (0.93, 1.48)		1.20 (0.94, 1.53)	
IGF1 rs6220				0.45			0.063		0.12 (0.47)			0.27		0.36 (0.59)
A/A	283	163 (66%)	85 (34%)		9.9 (9.1, 10.4)	1 (Reference)		1 (Reference)		23.9 (21.9, 27.1)	1 (Reference)		1 (Reference)	
Any G	262	146 (62%)	89 (38%)		10.5 (9.6, 11.7)	0.84 (0.70, 1.01)		0.86 (0.71, 1.04)		26.5 (23.5, 30.6)	0.89 (0.72, 1.10)		0.90 (0.73, 1.12)	
IGF1 rs2946834				0.85			***0.004***		***0.010 (0.074)***			0.12		***0.038*** (0.23)
C/C	257	143 (64%)	80 (36%)		9.7 (8.8, 10.3)	1 (Reference)		1 (Reference)		23.7 (20.8, 26.5)	1 (Reference)		1 (Reference)	
Any T	280	160 (63%)	93 (37%)		10.5 (9.6, 12.2)	0.77 (0.64, 0.92)		0.78 (0.64, 0.94)		28.0 (23.8, 30.8)	0.85 (0.69, 1.04)		0.79 (0.64, 0.99)	
IGFR1 rs2016347				1.00			0.56		0.35 (0.35)			0.22		***0.017*** (0.12)
T/T	160	90 (64%)	51 (36%)		9.7 (8.0, 10.1)	1 (Reference)		1 (Reference)		23.7 (19.4, 27.1)	1 (Reference)		1 (Reference)	
Any G	379	216 (64%)	121 (36%)		10.3 (9.4, 10.8)	0.94 (0.77, 1.15)		0.91 (0.74, 1.11)		25.1 (23.1, 28.5)	0.87 (0.69, 1.09)		0.75 (0.59, 0.95)	

(* *p* values was based on Fisher's exact test for tumor response, log-rank test for PFS and OS in the univariate analysis (†) and Wald test for PFS and OS in the multivariable Cox regression model adjusted for sex, ECOG performance status (0 *vs*. 1), primary tumor site (right *vs*. left), liver only disease (yes *vs*. no), number of metastases ($\leq$1, 2 and $>$2), RAS status (wildtype *vs*. mutant), BRAF status (wildtype *vs*. mutant), T stage ($>$2 *vs*. $\leq$2), and stratified by treatment arm (arm A *vs*. arm B) (‡); *p* values adjusted for multiple testing were shown in parentheses.).

Table 2. Association of SNPs and outcome in *KRAS* wild type patients (Arm A + B)

	Tumor response				Progression-free survival					Overall survival				
	N	Yes	No	*p* value*	Median (95%CI), months	Univariate HR (95%CI) †	*p* value*	Multivariable HR (95%CI) ‡	*p* value*	Median (95%CI), months	Univariate HR (95%CI) †	*p* value*	Multivariable HR (95%CI) ‡	*p* value*
IRS1 rs1801278				0.76			0.23		***0.036*** (0.22)			0.98		0.96 (1.00)
C/C	412	248 (67%)	123 (33%)		9.8 (9.2, 10.3)	1 (Reference)		1 (Reference)		26.1 (23.7, 28.7)	1 (Reference)		1 (Reference)	
Any T	67	37 (70%)	16 (30%)		12.5 (10.9, 13.5)	0.84 (0.63, 1.12)		0.73 (0.54, 0.98)		27.6 (21.3, 37.1)	1.00 (0.71, 1.39)		1.01 (0.71, 1.43)	
IRS1 rs1801123				0.34			0.51		0.78 (1.00)			***0.025***		***0.022*** (0.16)
T/T	391	240 (69%)	109 (31%)		10.1 (9.6, 11.1)	1 (Reference)		1 (Reference)		27.6 (24.7, 30.3)	1 (Reference)		1 (Reference)	
Any C	85	46 (63%)	27 (37%)		9.8 (8.6, 11.7)	1.09 (0.84, 1.40)		1.04 (0.80, 1.35)		21.3 (18.1, 25.4)	1.38 (1.04, 1.85)		1.41 (1.05, 1.90)	
IRS2 rs2289046				1.00			0.93		0.33 (0.80)			0.66		0.52 (1.00)
T/T	218	129 (67%)	63 (33%)		10.3 (9.3, 11.1)	1 (Reference)		1 (Reference)		23.8 (21.2, 29.1)	1 (Reference)		1 (Reference)	
Any C	250	150 (67%)	73 (33%)		10.0 (9.2, 10.9)	0.99 (0.81, 1.21)		0.90 (0.74, 1.11)		27.5 (23.9, 29.6)	0.95 (0.75, 1.20)		0.92 (0.73, 1.18)	
IRS2 rs1805097				0.52			0.46		0.10 (0.45)			0.73		0.53 (1.00)
A/A	213	124 (66%)	63 (34%)		10.1 (9.2, 11.1)	1 (Reference)		1 (Reference)		24.5 (21.2, 30.0)	1 (Reference)		1 (Reference)	
Any G	238	147 (70%)	64 (30%)		10.0 (9.3, 11.3)	0.93 (0.76, 1.13)		0.84 (0.68, 1.03)		27.1 (23.1, 29.1)	0.96 (0.76, 1.22)		0.92 (0.72, 1.18)	
IGF1 rs6214				0.91			0.34		0.38 (0.62)			0.19		0.090 (0.36)
G/G	163	96 (67%)	47 (33%)		9.6 (8.5, 10.6)	1 (Reference)		1 (Reference)		27.5 (23.2, 32.9)	1 (Reference)		1 (Reference)	
Any A	231	142 (68%)	67 (32%)		10.6 (9.9, 12.2)	0.90 (0.72, 1.12)		0.90 (0.72, 1.13)		24.5 (21.2, 28.1)	1.19 (0.92, 1.55)		1.27 (0.96, 1.67)	
IGF1 rs6220				0.60			***0.045***		0.13 (0.49)			0.24		0.31 (0.74)
A/A	244	145 (69%)	66 (31%)		9.9 (9.2, 10.6)	1 (Reference)		1 (Reference)		24.5 (21.9, 28.4)	1 (Reference)		1 (Reference)	
Any G	215	128 (66%)	66 (34%)		10.8 (9.6, 12.2)	0.82 (0.67, 1.00)		0.85 (0.69, 1.05)		27.6 (24.2, 31.9)	0.87 (0.68, 1.10)		0.88 (0.69, 1.13)	
IGF1 rs2946834				1.00			***<0.001***		***<0.001 (0.007)***			0.085		***0.040*** (0.24)
C/C	221	129 (67%)	63 (33%)		9.8 (8.9, 10.3)	1 (Reference)		1 (Reference)		23.7 (21.2, 27.1)	1 (Reference)		1 (Reference)	
Any T	228	137 (67%)	67 (33%)		11.1 (9.8, 12.8)	0.68 (0.56, 0.84)		0.70 (0.56, 0.86)		29.1 (24.8, 33.2)	0.81 (0.64, 1.03)		0.77 (0.60, 0.99)	
IGFR1 rs2016347				0.73			0.58		0.35 (0.73)			0.52		0.079 (0.38)
T/T	135	79 (66%)	41 (34%)		9.7 (8.0, 10.2)	1 (Reference)		1 (Reference)		25.2 (19.9, 28.6)	1 (Reference)		1 (Reference)	
Any G	314	187 (68%)	89 (32%)		10.4 (9.6, 11.5)	0.94 (0.75, 1.17)		0.90 (0.71, 1.13)		26.5 (23.6, 31.5)	0.92 (0.71, 1.19)		0.78 (0.60, 1.03)	

(* *p* values was based on Fisher's exact test for tumor response, log-rank test for PFS and OS in the univariate analysis (†) and Wald test for PFS and OS in the multivariable Cox regression model adjusted for sex, ECOG performance status (0 *vs*. 1), primary tumor site (right *vs*. left), liver only disease (yes *vs*. no), number of metastases ($\leq$1, 2, and $>$2), RAS status (wildtype *vs*. mutant), BRAF status (wildtype *vs*. mutant), T stage ($>$2 *vs*. $\leq$2), and stratified by arm (‡); *p* values adjusted for multiple testing were shown in parentheses.).

Table 3. Association of SNPs and outcome in *RAS* wild type patients (Arm A + B)

	Tumor response				Progression-free survival					Overall survival				
	N	Yes	No	*p* value*	Median (95%CI), months	Univariate HR (95%CI) †	*p* value*	Multivariable HR (95%CI) ‡	*p* value*	Median (95%CI), months	Univariate HR (95%CI) †	*p* value*	Multivariable HR (95%CI) ‡	*p* value*
IRS1 rs1801278				0.86			0.26		0.078 (0.33)			0.90		0.74 (1.00)
C/C	322	201 (69%)	89 (31%)		9.9 (9.2, 10.5)	1 (Reference)		1 (Reference)		27.5 (23.9, 29.8)	1 (Reference)		1 (Reference)	
Any T	58	32 (71%)	13 (29%)		12.5 (10.9, 14.1)	0.83 (0.61, 1.14)		0.75 (0.54, 1.03)		31.5 (22.5, 38.7)	1.02 (0.71, 1.48)		1.07 (0.72, 1.58)	
IRS1 rs1801123				0.65			0.65		0.70 (1.00)			***0.042***		***0.019*** (0.12)
T/T	309	192 (70%)	82 (30%)		10.4 (9.7, 11.5)	1 (Reference)		1 (Reference)		28.6 (25.0, 32.4)	1 (Reference)		1 (Reference)	
Any C	70	41 (67%)	20 (33%)		10.2 (8.6, 12.2)	1.07 (0.80, 1.41)		1.06 (0.79, 1.41)		21.8 (18.1, 30.8)	1.39 (1.01, 1.90)		1.48 (1.07, 2.05)	
IRS2 rs2289046				0.34			0.93		0.28 (0.73)			0.72		0.28 (0.48)
T/T	175	104 (67%)	52 (33%)		10.6 (9.7, 11.5)	1 (Reference)		1 (Reference)		25.2 (21.9, 32.4)	1 (Reference)		1 (Reference)	
Any C	197	124 (72%)	49 (28%)		9.9 (9.1, 11.8)	0.99 (0.79, 1.24)		0.88 (0.70, 1.11)		28.0 (24.5, 30.8)	0.95 (0.73, 1.24)		0.86 (0.65, 1.13)	
IRS2 rs1805097				0.18			0.52		***0.035*** (0.18)			0.57		0.11 (0.43)
A/A	169	99 (66%)	50 (34%)		10.5 (9.7, 11.7)	1 (Reference)		1 (Reference)		24.8 (21.2, 32.9)	1 (Reference)		1 (Reference)	
Any G	191	124 (74%)	44 (26%)		10.2 (9.2, 12.2)	0.93 (0.74, 1.17)		0.77 (0.61, 0.98)		27.5 (24.2, 30.6)	0.93 (0.71, 1.21)		0.80 (0.60, 1.06)	
IGF1 rs6214				0.79			0.60		0.55 (1.00)			0.084		***0.045*** (0.23)
G/G	137	83 (69%)	37 (31%)		9.7 (8.7, 11.1)	1 (Reference)		1 (Reference)		29.0 (24.5, 33.8)	1 (Reference)		1 (Reference)	
Any A	184	119 (71%)	48 (29%)		10.5 (9.9, 12.3)	0.94 (0.74, 1.20)		0.93 (0.72, 1.19)		24.7 (20.7, 28.7)	1.29 (0.96, 1.73)		1.37 (1.01, 1.86)	
IGF1 rs6220				0.72			***0.024***		***0.034*** (0.21)			0.30		0.16 (0.41)
A/A	198	122 (71%)	49 (29%)		9.9 (9.2, 10.6)	1 (Reference)		1 (Reference)		25.6 (22.7, 28.8)	1 (Reference)		1 (Reference)	
Any G	170	106 (69%)	47 (31%)		11.3 (9.6, 13.0)	0.77 (0.62, 0.97)		0.78 (0.61, 0.98)		29.1 (24.5, 33.8)	0.87 (0.67, 1.14)		0.82 (0.62, 1.08)	
IGF1 rs2946834				0.71			***<0.001***		***<0.001 (0.002)***			***0.037***		***0.003 (0.024)***
C/C	177	105 (69%)	47 (31%)		9.9 (8.8, 10.4)	1 (Reference)		1 (Reference)		23.7 (20.3, 27.5)	1 (Reference)		1 (Reference)	
Any T	184	118 (71%)	48 (29%)		11.8 (10.0, 13.2)	0.65 (0.51, 0.81)		0.63 (0.50, 0.81)		30.8 (26.4, 35.0)	0.75 (0.58, 0.99)		0.65 (0.49, 0.87)	
IGFR1 rs2016347				0.59			0.90		0.49 (0.86)			0.86		0.12 (0.39)
T/T	108	64 (67%)	31 (33%)		9.9 (8.7, 10.9)	1 (Reference)		1 (Reference)		26.4 (23.1, 29.1)	1 (Reference)		1 (Reference)	
Any G	254	158 (71%)	65 (29%)		10.5 (9.6, 11.8)	0.98 (0.77, 1.26)		0.91 (0.70, 1.18)		27.5 (23.7, 32.9)	0.98 (0.73, 1.30)		0.78 (0.57, 1.06)	

(* *p* values was based on Fisher's exact test for tumor response, log-rank test for PFS and OS in the univariate analysis (†) and Wald test for PFS and OS in the multivariable Cox regression model adjusted for sex, ECOG performance status (0 *vs*. 1), primary tumor site (right *vs*. left), liver only disease (yes *vs*. no), number of metastases ($\leq$1, 2, and $>$2), RAS status (wildtype *vs*. mutant), BRAF status (wildtype *vs*. mutant), T stage ($>$2 *vs*. $\leq$2), and stratified by arm (‡); *p* values adjusted for multiple testing were shown in parentheses.) .

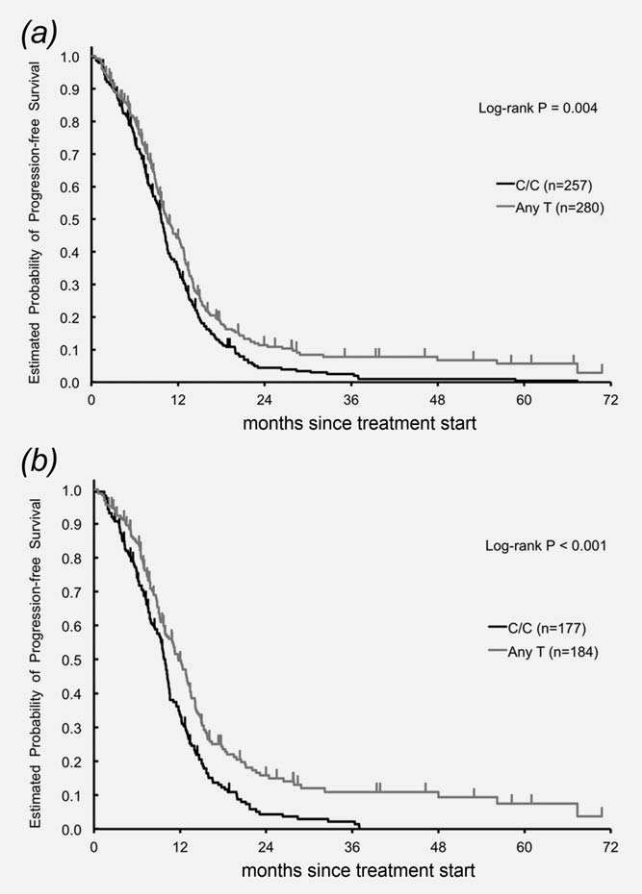

Figure 2. (*a*) IGF1 rs2946834 and PFS in overall population of FIRE 3 trial. (*b*) IGF1 rs2946834 and PFS in RAS wild type patients.

expression appears inversely correlated to CRC differentiation and it shows higher immunostaining levels in hepatic metastases compared to both primary CRC and paired colonic epithelium. All the above-mentioned studies lead to hypothesize a role for *IRS*-1 in CRC progression and development of liver metastasis.[10] *IRS1* rs1801123 was also significantly associated with lymph node involvement in a cohort of breast cancer patients with locally invasive tumor and estrogen receptor sensitivity.[22]

IRS1 is considered a marker of active *IGF-1* signaling pathways in tumors and its phosphorylation mediated by *IGF-1R*, transmit mitogenic, anti-apoptotic, and anti-differentiation signals to the cell through the *PI3K* and the *ERK* pathways.[23] *IRS1* is constitutively activated in many human tumors[24] and it's critical for tumor growth, it has been demonstrated that down regulation of *IRS1* (by antisense or siRNA procedures) reverses the transformed cancer phenotype.[25] Moreover it has been implicated in resistance to EGFR and mTOR inhibitors.[26]

Recently a new family of *IGF-IR/IRS*-targeted agents named the NT compounds has been developed and showed promising preclinical activity in the inhibition of IRS proteins. In particular they act as competitive inhibitors of IGF-IR, promote inhibitory phosphorylation and degradation of *IRS1* and *IRS2* leading to the elimination of *IRS1/2* and finally to cell death.[27] Among NT compounds, NT157 showed to prevent colorectal tumor development and caused durable regression of established tumors and attenuated metastatic growth in murine models and in human CRC cells.[28]

Explorative subgroup analyses were performed in order to identify a possible predictive role for the selected SNPs and a prognostic role in a molecularly selected population of RAS wild type patients. No specific associations were identified according to treatment arm, suggesting no predictive effect for *IRS1* and *IRS2* SNPs to anti-EGFRs. However the interesting observation of a prognostic role for *IRS1 SNPs* in *RAS* wild type patients strengthens the biological rationale of our findings confirms the relation between the IGF-IGF-1R-IRS with the EGFR signaling pathway and underlines the importance of *IRS1* as a new possible target in *RAS* wild type mCRC patients. Although we have to admit that the statistical significance was lost when applying correction for multiple testing, our results are still relevant as hypothesis generating findings and might be validated in future trials.

As limitation of our results, due to the exploratory nature of those findings and to the unavailability of adequate tissue samples, we did not perform additional expression analyses aiming at the identification of possible correlations between *IRS1* rs1801123 C/T SNP and IRS1 expression. *IRS1* rs1801123 C/T SNP has been associated with splicing and post-transcriptional regulation of IRS1 protein, we can hypothesize that *IRS1* rs1801123 C allele might be associated with higher IRS1 expression and worse prognosis in CRC patients.

On the other hand, we prospectively confirmed a prognostic role for *IGF1* rs2946834 in the overall population of FIRE-3 trial as well in the subgroup of molecularly selected *RAS* wild type patients. In our previous work multivariable models identified a significant association with PFS for IGF rs6214, *IGF1* rs6220 and *IGF1* rs2946834 and a significant association with OS for *IGFR1* rs2016347 in the overall population of chemo-refractory mCRC patients treated with cetuximab monotherapy in the IMC-0144 trial.[29] An association with the subgroup of *KRAS* wildtype patients and PFS was observed for *IGF1* rs6214 and *IGF1* rs2946834. The lack of a control group and the tight connection of the *IGF-IGFR1* with the EGFR downstream signaling pathway allowed us to hypothesize a possible predictive role of cetuximab efficacy for those SNPs.[22] In the present study, the predictive effect was denied and the prognostic role was confirmed since *IGF1* rs2946834 predicted PFS in patients treated with FOLFIRI plus cetuximab and in those treated with FOLFIRI plus bevacizumab. The connection of *IGF1* rs2946834 and *rs6220* with *RAS* status in the prediction of mCRC prognosis was also confirmed. Such findings are supported by a strong biological rationale: *IGF1* rs2946834 SNP is located in the 3′UTR of the *IGF1* gene and affect mRNA stability and translational expression. This SNP has been related with circulating levels

of IGF1 in breast cancer patients and high levels of IGF1 correlated with poor outcome in CRC and response to IGF-1R inhibition.[30–32] From a methodological point of view, the prospective verification of our previous findings further enforces the reliability of our results and underline the need of prospective validation of results obtained from genotyping studies.[33]

From a more general perspective, the identification of specific prognostic factors in *RAS* wild type patients is crucial. Nowadays it is well known that it is not possible to refer to CRC patients as a homogeneous population since cases carrying *RAS* wild type have specific clinical characteristics, outcome and derive different benefit from treatment compared to *RAS* mutant.[34–37] Our results confirm that *IGF-IGFR1* pathway affects specifically *RAS* wild type tumors. Among *RAS* wild type patients *IGF1* and *IRS1* SNPs might allow to identify specific subgroup of patients.

IGF signaling has been proposed as an attractive target in the last few years for the development of novel anticancer therapeutics.[38] However, despite promising biological rationale and preclinical data, studies failed to provide definitive evidence that *IGF-1R* may represent a valid therapeutic target in solid tumors.[30,31,39–42] As an example, in chemorefractory *KRAS* wild type mCRC patients, the phase II/III trial of dalotuzumab, an anti-IGF-1R monoclonal antibody, in combination with cetuximab and irinotecan failed to demonstrate an improvement of OS.[31,32] Possibly, patients selection was not adequate and that might have influenced results of clinical trials.[43] Moreover it has been hypothesized that only in a minority of cases IGF-1R is activated and plays a crucial role.[44] Our results, if validated in clinical trials might allow identifying *IRS1* and *IGF1* SNPs as useful tool in order to select patients who benefit from anti- IGF-1R drugs.

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