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Improving selection of patients with metastatic colorectal cancer to benefit from cetuximab based on KIR genotypes

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ABSTRACT

Aim Cetuximab is a standard-of-care treatment for *KRAS* wild-type metastatic colorectal cancer (mCRC), but it may also be effective in a subgroup of *KRAS* mutant patients by its immunomodulatory activity. Here, we explore if KIR (killer cell immunoglobulin-like receptor) genotyping can provide a significant added value in the clinical outcome of patients with *KRAS* mutant mCRC based on cetuximab treatment.

Methods We included 69 patients with histologically confirmed mCRC and *KRAS* mutation, positive EGFR expression, and Eastern Cooperative Oncology Group performance status ≤2. Based on KIR gene content, haplotype (A or B) was defined and genotypes (AA or Bx) were grouped for each patient.

Results We demonstrated with new evidence the immunomodulatory activity of cetuximab in patients with *KRAS* mutant mCRC. Patients with homozygous genotypes (AA or BB) showed shorter 12-month progression-free survival (PFS12) and poorer overall survival (OS) than those with heterozygotes (AB). Moreover, multivariate analysis confirmed stratification of patients based on genotype was an independent marker of PFS12 (HR 2.16) and the centromeric and telomeric distribution of KIRs was an independent predictor of both PFS12 (HR 2.26) and OS (HR 1.93) in patients with mCRC with *KRAS* mutation treated with cetuximab.

Conclusions Selection of patients with mCRC based on their KIR genotypes opens a therapeutic opportunity for patients with *KRAS* mutation, and it should be tested in clinical trials in comparison with other alternatives with scarce benefit.

Trial registration number NCT01450319, EudraCT 2010-023580-18.

INTRODUCTION

In 2018, almost 2 million new cases of colorectal cancer (CRC) and nearly 900,000 deaths were estimated. Overall, this cancer ranks third in terms of incidence but second in terms of mortality.[1] At the time of diagnosis, about 75% of patients with CRC show metastases and surgery is not possible in most cases.[2]

In these patients, monoclonal antibodies (mAbs) are the most widely used targeted therapies, such as cetuximab, which inactivates Epidermal Growth Factor Receptor (EGFR) signaling. Cetuximab acts as a functional antagonist by blocking ligand binding to EGFR and therefore inhibits EGFR activation and downstream signaling in tumor cells, preventing cell proliferation, angiogenesis and metastatic tendencies.[3 4] Unfortunately, it is not effective in all patients due to resistance to this therapy.[5–9]

Among the major downstream signaling activated by EGFR, the RAS-RAF-MAPK, PI3K-PTEN-AKT, and JAK/STAT pathways have been described as resistance mechanisms to antibody-mediated EGFR blocking. Any change in these elements, such as *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* gene mutations, can lead to constitutive activation of EGFR, causing drug resistance.[10] Therefore, the status of *KRAS* in the tumor affects response to cetuximab. It is demonstrated that this therapy is effective in patients with wild-type *KRAS* CRC; however, some studies have determined that, although the majority of cases of *KRAS* mutant CRC do not respond to cetuximab, some subjects could be sensitive under certain circumstances,[11–13] suggesting that other mechanisms of action apart from EGFR blockage could be involved.[14] The identification of this subgroup of patients is very relevant since 36%–46% of patients with metastatic colorectal cancer (mCRC), depending on tumor sidedness, have mutations in *KRAS*.[15]

Natural killer (NK) cells are an important subset of lymphocytes in the defense of organisms against viral infection and the development of tumors. They express many activating and inhibiting receptors that regulate their function by an equilibrium between incoming activating and inhibitory signals.[16] When NK cells encounter a target cell, these signals are integrated and a response is formed immediately.[17]

As an IgG1 isotype mAb, cetuximab has two functional motifs: one is that it is able to bind to the extracellular domain of the EGFR and the other is that it can induce immune functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), involving CD16 receptors present in NK cells.[3]

The antitumor activity of NK cells is not only mediated by CD16, but also by other receptors such as the KIR (killer cell immunoglobulin-like receptor) encoded by different genes (*2DL1*, 2*DL2*, *2DL3*, *2DL4*, *2DL5*, *2DS1*, *2DS2*, 2DS3, *2DS4*, *2DS5*, *3DL1*, *3DL2*, *3DL3* and *3DS1*) and pseudogenes (*2DP1* and *3DP1*). They can also be either activating or inhibitory and thereby are critical in the determination of NK cell activation.[18] Therefore, the NK activity in patients with cancer treated with cetuximab is dependent not only on CD16, but also on the balance of signals from activating and inhibitory receptors on the NK cell surface such as the KIR.

The KIR region shows high genomic diversity mainly due to the fact that each person can have a variable number of the genes aforementioned, and it allows us to describe different haplotypes, semihaplotypes and genotypes.[19 20] In each of them we can define subtype A or subtype B if the inhibitor or activator genes predominate, respectively. The goal of this variability is probably the diversification of the immune response in the context of a rapidly changing environment. Therefore the identification of KIR genotypes will help to elucidate the influence of these genes on different disease states such as cancer.[21]

The role of KIR haplotypes and genotypes in patients with *KRAS* mutant mCRC receiving cetuximab has not been studied. However, it is known that the interaction of KIRs with their human leukocyte antigen (HLA) ligands and CD16 through its fragment crystallizable (Fc) portion of IgG1 isotype antibodies can activate NK effector cells. Thus, the combined action of both may be translated into a superior antitumor effect of cetuximab.[22 23] In this context, it seems reasonable that depending on the KIR genotype the response to cetuximab can be modified, since the final result of the NK response depends on the balance of activating and inhibitory signals.

We have previously demonstrated that patients with mCRC harboring FcγRII H131 and non-functional variant of KIR2DS4 showed a significant benefit in terms of prolonging time to progression and overall survival (OS).[24 25] In this research, we explore if the use of the KIR genotypes and semihaplotypes could improve the identification of patients with *KRAS* mutant mCRC that would derive a benefit from cetuximab therapy.

MATERIALS AND METHODS

Study cohort

The design and the methods of this multicenter, phase II clinical trial have been previously reported,[25] and a summary of the methods follows. Clinical data and biospecimens were collected from patients with mCRC enrolled between September 2011 and December 2013. Written informed consent was obtained from all patients before enrollment. Eligibility included patients 18 years or older with mCRC with verified *KRAS* mutation (all patients, except one, showed mutation in *KRAS* G12), positive EGFR (immunohistochemistry determination), carrier of polymorphism H131 allele in FcγRIIa and Eastern Cooperative Oncology Group (ECOG) performance status of 0–2. In our study, analyses were restricted to patients who had informative KIR genes to perform haplotype analyses (N=69). Details of patient recruitment are shown in online supplemental figure 1.

Baseline measurements

Several prognostic factors have been described in CRC, such as blood levels of carcinoembryonic antigen (CEA), lactate dehydrogenase (LDH) and β2 microglobulin.[26–28] Therefore, they were evaluated before initiation of cetuximab therapy. Patients were grouped according to the upper limit of the normal range for each measure (>5 µg/L for CEA, 333 UI/L for LDH and 3 mg/L for β2 microglobulin).

KIR genotyping

To perform this analysis we used the previously determined presence or absence of KIR genes.[25] Briefly, KIR Genotyping SSP Kit (Applied Biosystems) was used to determine the 17 KIR genes. Genotype-specific PCR products were amplified and later resolved using agarose gels, and the interpretation of the results was performed following the manufacturer's instructions.

Based on gene content, haplotype (A or B) was defined and genotypes (AA or Bx, where X can be A or B) were grouped for each patient. Genotype AA is homozygous for A inhibitory haplotype (formed by 3DL3, 2DL3, 2DP1, 2DL1, 3DP1, 2DL4, 3DL1, 2DS4 and 3DL2). Bx genotype could be heterozygous (A and B haplotypes) or homozygous for B activator haplotype (constituted by 3DL3, 2DS2, 2DL2, 2DL5B, 2DS3, 2DP1, 2DL1, 3DP1, 2DL4, 3DS1, 2DL5A, 2DS3/2DS5, 2DS1 and 3DL2).

KIR haplotypes consist of two regions, centromeric (CEN) and telomeric (TEL), with different gene content. According to the presence or absence of one or more B haplotype-defining KIR genes, the genotypes for the CEN and TEL parts were defined. Thus, cenA (cA) is the CEN motif and telA (tA) is the TEL motif of the A haplotype; cenB (cB) and telB (tB) are the CEN and TEL motifs of the B haplotype, respectively.[29] cenA is defined by centromeric 2DL3, cenB is composed of centromeric 2DS2, 2DL2, 2DL5B and 2DS3, telA is determined by telomeric 3DL1 and 2DS4, and telB is defined by telomeric 3DS1, 2DL5A, 2DS5 and 2DS1.

KIR B score

KIR B-content score is defined as the number of CEN and TEL gene content motifs having B haplotype-defining genes. It is calculated by adding the number of cenB and/or telB motifs in each genotype. Permissible values for the KIR B-content score are 0, 1, 2, 3, and 4.[29] Additionally, a

classification of KIR B status was performed: best (score ≥2, cenB/B and any tel), better (score ≥2, cenA/x and telB/x) and neutral (score 0–1).[29]

Statistical methods

Survival studies were carried out by Kaplan-Meier curves as well as Cox regression analysis. Twelve-month progression-free survival (PFS12) was defined as the time of survival until progression or death from any cause within 12 months after initial therapy. The OS period (months) was calculated from patient recruitment date until death due to any cause or last follow-up, when appropriate. Patients alive at last follow-up or those who started a new therapeutic regimen (differing from cetuximab) were censored. In such case, those patients were censored at the date of beginning a new drug scheme.

Kaplan-Meier curves as well as univariate and multivariate Cox regression study were developed to evaluate the impact of KIR genotypes, haplotypes and semihaplotypes on survival. Age and gender were included in the multivariate analysis as confounding variables. Data analysis was carried out with SPSS statistics software (V.20.0).

RESULTS

Patient characteristics and clinical outcome

The demographic and disease characteristics of the cohort are shown in table 1. The median time of follow-up was 6.4 months (IQR 3.7–10.1 months). Fifty-four (78.3%) patients died during the study period, with a median OS of 6.30 (95% CI 3.4 to 8.2).

KIR haplotype frequencies

Tables 2–4 show the genotype, semihaplotype and KIR B-content frequencies from the series of this study. All of them were included in the survival analysis.

The genotype distribution in the study population was 33 (47.8%) AB heterozygotes, 20 (29%) homozygotes for the B haplotype, and 16 (23.2%) homozygotes for the A haplotype (table 2). Since the gene content is different in the CEN and TEL regions, genotypes AA and Bx were examined based on their distribution. Four different combinations were found. The cAcA-tAtA was found in 23% of patients, cAcA-tBx and cBx-tAtA were observed in 22% and 20%, respectively, and cBx-tBx was found in 32% of patients; 2 (2.9%) patients were not informative (table 3). Regarding KIR B-content score, 39 patients showed scores of 0–1, 25 showed a score of 2, and 5 patients showed scores of 3–4 (table 4).

Survival analysis

Of the clinical and biochemical parameters included in the study, the number of metastatic sites was significantly associated with increased risk of death by univariate analysis (table 5).

The prognostic impact of specific known *KRAS* codon 12 mutations (G12C, G12V and G12D) on survival in patients with CRC has been previously described.[30 31]

Table 1 Baseline characteristics of patients included in the study

Variable	Patients (N=69)
Age at diagnosis, years, median (IQR)	64 (5.5–72.5)
Gender, n (%)	
Male	35 (50.7)
Female	34 (49.3)
Primary site, n (%)	
Colon	51 (73.9)
Rectum	18 (26.1)
Laterality, n (%)	
Right-sided	16 (23.2)
Left-sided	53 (76.8)
Number of metastatic sites, n (%)	
1	26 (37.7)
2	27 (39.1)
3 or more	16 (23.2)
ECOG performance status, n (%)	
0	12 (17.4)
1	51 (73.9)
2	6 (8.6)
CEA basal, n (%)	
≤ULN	7 (10.1)
≥ULN	60 (87)
N/A	2 (2.9)
LDH basal, n (%)	
≤ULN	34 (49.3)
≥ULN	30 (43.5)
N/A	5 (7.2)
β2 microglobulin basal, n (%)	
≤ULN	52 (75.4)
≥ULN	8 (11.6)
N/A	9 (13)

CEA, carcinoembryonic antigen; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; N/A, not available; ULN, upper limit of the normal range.

Therefore, we assessed the impact of mutations at *KRAS* codon 12 on PFS12 and OS in our cohort. Significant differences in PFS12 or OS were not found between patients carrying p.G12C, p.G12V or p.G12D point mutations and those who carried any of the rest of the mutations, nor was any significant difference observed when mutations were stratified by transitions (p.G12D, p.G12R and p.G12S) and transversions (p.G12A, p.G12C and p.G12V) (online supplemental figure 2).

Regarding KIR genotypes, Cox regression analysis showed significant associations with PFS12 and OS (tables 6 and 7).

Table 2 Genotypes based on A and B haplotypes

Genotype	Frequency (%)
AA	16 (23.2)
AB	33 (47.8)
BB	20 (29)

Homozygous patients carrying genotype AA or BB showed significantly lower PFS12 (p=0.01, log-rank test) (figure 1A) and OS (p=0.048, log-rank test) (figure 2A) than heterozygotes AB. When homozygous patients were grouped (AA+BB) and compared with heterozygotes (AB), the difference in Kaplan-Meier curves was more significant for both PFS12 (p=0.003, log-rank test) (figure 1B) and OS (p=0.015, log-rank test) (figure 2B), supporting homozygotes have poorer survival than heterozygotes. Genotypes AA and Bx were analyzed based on the CEN and TEL regions; cAcA-tAtA or cBx-tBx carriers had lower PFS12 (p=0.002, log-rank test) (figure 1C) and OS (p=0.006, log-rank test) (figure 2C) than cAcA-tBx or cBx-tAtA carriers. Moreover, genotype AA showed lower PFS12 than genotype Bx/cenA (p=0.026, log-rank test) (figure 1D).

Multivariate logistic regression model, adjusted for number of metastatic sites and confounding variables (age and gender), showed patients with AA or BB genotypes had a twofold increased risk of progression (HR 2.16, 95% CI 1.26 to 3.78, p=0.005) compared with AB heterozygous (table 6) and confirmed this variable as an independent marker. Moreover, stratification of patients based on CEN and TEL distribution also showed that both CEN genotype and CEN–TEL genotype were independent predictors of PFS12 (table 6), with the latter also an independent predictor of OS in patients with *KRAS* mutated mCRC receiving cetuximab (table 7). cAcA-tAtA or cBx-tBx carriers showed a twofold increased risk of progression (HR 2.26, 95% CI 1.29 to 3.97, p=0.005) and death (HR 1.93, 95% CI 1.02 to 3.65, p=0.044) than cAcA-tBx or cBx-tAtA carriers.

Table 3 Distribution of variables in KIR centromere–telomere genotype

Centromere–telomere	Frequency (%)
Centromere	
cAcA	30 (43.5)
cBx	37 (53.6)
N/A	2 (2.9)
Telomere	
tAtA	30 (43.5)
tBx	37 (53.6)
N/A	2 (2.9)
Centromere–telomere	
cAcA-tAtA	16 (23.2)
cAcA-tBx	15 (21.7)
cBx-tAtA	14 (20.3)
cBx-tBx	22 (31.9)
N/A	2 (2.9)

Bx, X can be A or B haplotype; cA, centromeric motif A haplotype; cB, centromeric motif B haplotype; KIR, killer cell immunoglobulin-like receptor; N/A, not available; tA, telomeric motif A haplotype; tB, telomeric motif of B haplotype.

Table 4 KIR B-content score and KIR B status based on score and centromeric/telomeric localization of activator KIR genes

B-content	Frequency (%)
Score	
0	16 (23.2)
1	23 (33.3)
2	25 (36.2)
3	4 (5.8)
4	1 (1.4)
Status	
Neutral	39 (56.5)
Better	23 (33.3)
Best	7 (10.1)

KIR, killer cell immunoglobulin-like receptor.

DISCUSSION

We have previously described higher OS in patients with *KRAS* mutant mCRC carrying the non-functional receptor KIR2DS4,[25] and we also identified that polymorphisms in Fc gamma receptors (FcγRs) contribute to differences in the immune response to cetuximab.[24] Now, this study provides new evidence that, in patients with *KRAS* mutant mCRC, cetuximab plays a primary role as an immunomodulatory treatment. Our results show how stratification of patients based on KIR genotypes (AA or BB vs AB) or based on CEN and TEL KIR distribution was an independent predictor of progression-free survival, with the latter also an independent biomarker of OS, with twofold increased risk of death for cAcA-tAtA or cBx-tBx carriers.

The immune-modulating effects of KIRs are regulated by the balance among inhibitors or activator signals, which depend on the number and type of receptors present in each individual.[32 33] In this regard, KIR genes and their combination define two main groups of haplotypes (A and B). The A haplotype predominates genes encoding inhibitory receptors, while B haplotypes are more heterogeneous in their composition, presenting a greater number of activating receptors.[34] Due to the evident strength of immunomodulatory activity of cetuximab, we hypothesized that KIR receptors and their genotype may influence the response of this treatment in patients with *KRAS* mutant mCRC.

Table 5 Univariate analysis of clinical variables for 12-month progression-free survival and overall survival

	Univariate PFS12		Univariate OS	
Variable	**HR (95% CI)**	**P value**	**HR (95% CI)**	**P value**
Age (continuous)	1.016 (0.991 to 1.042)	0.20	1.010 (0.98 to 1.04)	0.47
Gender				
Male	1		1	
Female	1.074 (0.663 to 1.739)	0.77	1.105 (0.65 to 1.88)	0.71
Primary site				
Rectum	1		1	
Colon	1.567 (0.901 to 2.727)	0.11	1.193 (0.65 to 2.17)	0.56
Laterality				
Right-sided	1		1	
Left-sided	1.114 (0.632 to 1.965)	0.71	1.373 (0.65 to 2.17)	0.34
Number of metastatic sites				
1	1		1	
2	1.277 (0.736 to 2.217)	0.38	1.265 (0.67 to 2.40)	0.47
3 or more	1.750 (0.910 to 3.367)	0.09	2.942 (1.463 to 5.915)	**0.002**
ECOG				
0	1		1	
1	1.037 (0.550 to 1.955)	0.91	1.178 (0.57 to 2.43)	0.66
2	1.154 (0.418 to 3.187)	0.78	1.100 (0.36 to 3.30)	0.87
CEA basal				
≤ULN	1		1	
≥ULN	1.064 (0.482 to 2.351)	0.87	1.151 (0.49 to 2.70)	0.75
LDH basal				
≤ULN	1		1	
≥ULN	1.173 (0.709 to 1.940)	0.53	1.157 (0.66 to 2.02)	0.61
β2 microglobulin basal				
≥ULN	1		1	
≤ULN	1.281 (0.603 to 2.722)	0.52	1.241 (0.58 to 2.67)	0.58

P-values numbers marked in bold indicate those that are lower than 0.05.

CEA, carcinoembryonic antigen; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; OS, overall survival; PFS12, 12-month progression-free survival; p-values in bold, indicate those that are lower than 0.05; ULN, upper limit of the normal range.

Despite the essential role of individual KIR genes in modulating NK cell function, as proven by our group,[35] the impact of the KIR genotypes on the outcome of patients with mCRC has not been sufficiently explored.

The association of different KIR genes and the susceptibility to develop CRC has been studied. In contrast to hematological malignancies, CRC seems to be affected by the hyperactivity of the NK cells.[36 37]

Even more interesting appears to be those studies that evaluate the effect of KIRs in different treatments. The anticancer treatments modify the tumor environment, the expression of HLA, and the release of tumor neoantigens, decreasing the tumor mass furthering as a whole that the immune system can be effective.[38 39]

The immunomodulatory effect of cetuximab has been studied in different types of cancers, including mCRC. Most of these studies have focused on the analysis of specific polymorphisms and their influence on ADCC response.[40–42] However, the interaction between NK cells and target cells is more complex, involving many more molecules, among which KIRs play an important role. The intrinsic mechanism by which KIRs modulate the anticancer effects in patients with *KRAS* mutant mCRC receiving cetuximab is unknown. Activation signals mediated by both KIR and CD16 could be necessary for NK activity. As NK cells require a positive balance of activating signals, the joint action of KIR and CD16 could enhance cytotoxic activity. Veluchamy *et al*[43] found that cetuximab enhanced NK cell cytotoxic activity in a CD16-dependent manner in EGFR-positive tumor cells regardless of the presence of *RAS* or *BRAF* mutations. These results support the importance of NK cell immunotherapy in

Table 6 Univariate and multivariate analyses for 12-month progression-free survival

	Univariate		Multivariate	
	HR (95% CI)	P value	HR (95% CI)	P value*
Genotype				
AB	1		1	
AA	2.27 (1.21 to 4.25)	**0.01**	2.58 (1.31 to 5.10)	**0.006**
BB	1.99 (1.12 to 3.55)	**0.02**	1.93 (1.04 to 3.58)	**0.03**
Genotype				
AB	1		1	
AA or BB	2.10 (1.28 to 3.47)	**0.004**	2.16 (1.26 to 3.78)	**0.005**
Centromere–telomere genotype				
cA/tB or cB/tA	1		1	
cA/tA or cB/tB	2.18 (1.29 to 3.67)	**0.003**	2.26 (1.29 to 3.97)	**0.005**
Centromere genotype				
Bx/cenA	1			
AA	2.31 (1.08 to 4.95)	**0.03**	2.50 (1.02 to 6.14)	**0.04**

*Multivariate analysis includes age, gender and number of metastases as covariates for 12-month progression-free survival; p≤0.05 denotes statistical differences.
Bx, X can be A or B haplotype; cA, centromeric motif A haplotype; cB, centromeric motif B haplotype; p-values in bold, indicate those that are lower than 0.05; tA, telomeric motif A haplotype; tB, telomeric motif of B haplotype.

combination with cetuximab for patients with *RAS* or *BRAF* mutant mCRC. Recently, the results obtained by Faden *et al*[44] strongly suggest that HLA-C–KIR interaction is indeed important for NK cell activation in patients treated with cetuximab, and alterations in HLA-C may be involved in the immune evasion mechanism by affecting this activation.

However, none of these studies evaluated the contribution of KIR genotypes, as has been assessed in this work. In this study, we have analyzed how complete KIR genotypes can influence the outcome of patients with *KRAS* mutant mCRC treated with cetuximab. This aspect may be of great interest since the final effect mediated by the KIRs depends mainly on the balance of inhibitory

Table 7 Univariate and multivariate analyses for overall survival

	Univariate		Multivariate	
	HR (95% CI)	P value	HR (95% CI)	P value*
Genotype				
AB	1			
AA	2.08 (1.04 to 4.15)	**0.04**	2.02 (0.97 to 4.2)	0.06
BB	1.89 (1 to 3.55)	**0.05**	1.58 (0.79 to 3.11)	0.19
Genotype				
AB	1			
AA or BB	1.96 (1.13 to 3.41)	**0.017**	1.74 (0.97 to 3.15)	0.06
Centromere–telomere genotype				
cA/tB or cB/tA	1		1	
cA/tA or cB/tB	2.20 (1.23 to 3.93)	**0.007**	1.93 (1.02 to 3.65)	**0.044**
Centromere genotype				
Bx/cenA	1			
AA	2.31 (0.99 to 5.37)	**0.05**	1.67 (0.61 to 4.56)	0.31

*Multivariate analysis includes age, gender and number of metastases as covariates for overall survival; p≤0.05 denotes statistical differences.
Bx, X can be A or B haplotype; cA, centromeric motif A haplotype; cB, centromeric motif B haplotype; p-values in bold, indicates those that are lower than 0.05; tA, telomeric motif A haplotype; tB, telomeric motif of B haplotype.

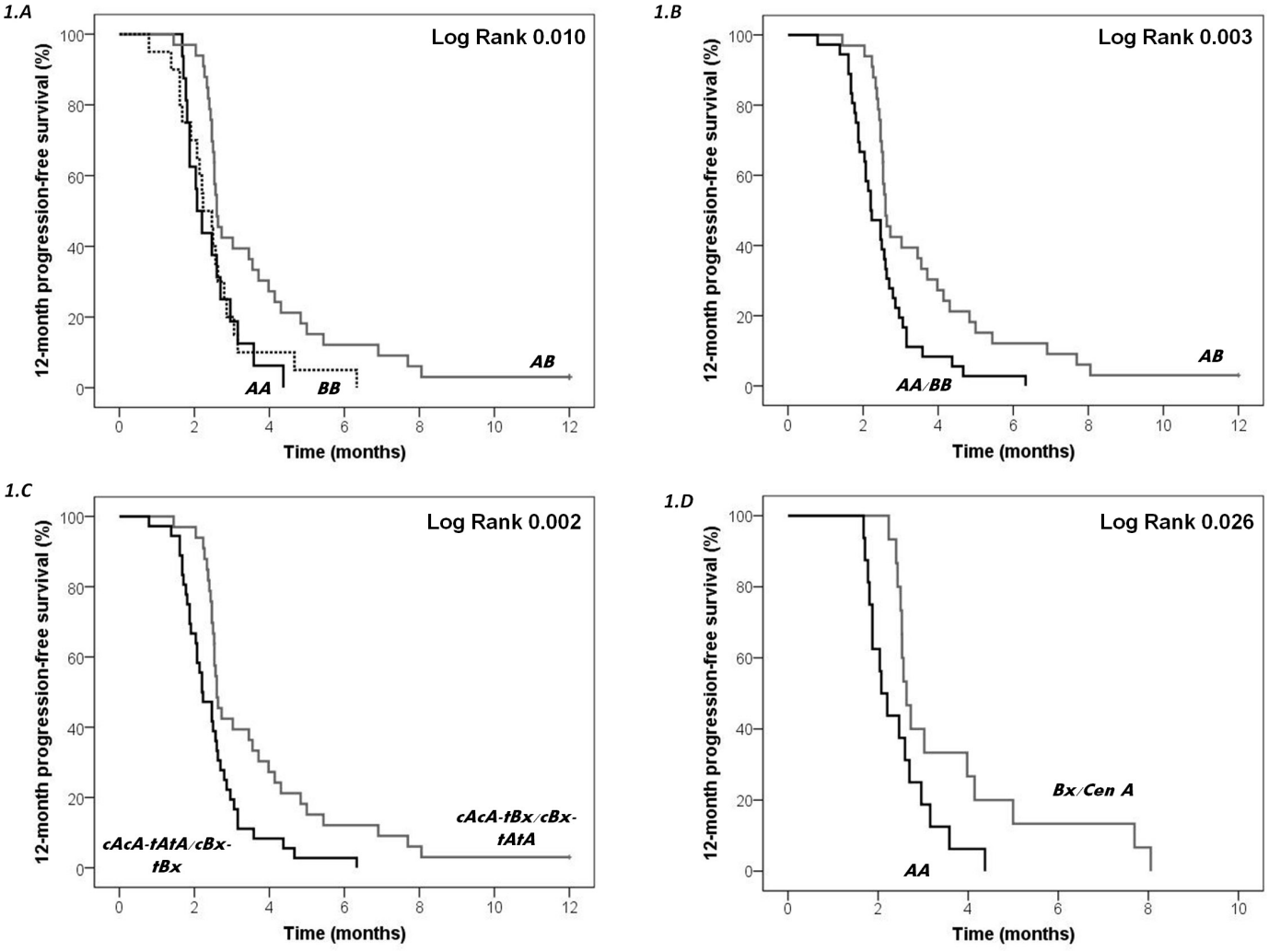

Figure 1 Kaplan-Meier curve for 12-month progression-free survival (PFS12) according to genotypes or centromeric and telomeric KIR content. Heterozygous individuals (AB) were compared with homozygotes (AA or BB) separately (A) or in combination (AA and BB) (B). The median PFS12 among homozygotes (AA+BB) was 2.21 months and among heterozygous patients 2.59 months (p=0.003, log-rank test). Genotypes (AA and Bx) were assessed based on the centromeric and telomeric regions (C) or only based on the centromeric region (D). The median PFS12 among homozygotes (cAcA-tAtA or cBx-tBx) was 2.28 months, whereas among heterozygotes (cAcA-tBx or cBx-tAtA) was 2.63 months (p=0.002, log-rank test). The median PFS12 among homozygotes (AA) was 2.13 months, whereas among heterozygotes (Bx/cenA) was 2.63 months (p=0.026, log-rank test). cenA (cA) and telA (tA), centromeric and telomeric motifs of the A haplotype; cenB (cB), centromeric motif of the B haplotype; KIR, killer cell immunoglobulin-like receptor; telB (tB), telomeric motif of the B haplotype.

and activating signals, as Braun *et al*[45] highlighted in their study where ethnicity and tuberculosis status were analyzed. They found that the KIR profile and haplotype were more predictive than the presence or absence of individual genes.

Similarly, Siebert *et al*[46] found that patients with neuroblastoma with B haplotype showed a higher level of ADCC and superior event-free survival (EFS) than those with A inhibitory haplotype. In bone marrow transplant, patients homozygous for A haplotype had an improved OS, higher EFS and non-relapse mortality when donors expressed at least one KIR B haplotype.[47] It has also been described that allogeneic transplant donors carrying a KIR B haplotype and lacking a recipient HLA-C epitope protect against relapse from acute myeloid leukemia.[48] Apart from these studies, there are few research analyzing the influence of specific combinations of KIR genotypes in the treatment response of solid cancers.

Our study indicates that patients with both an inhibitor haplotype and an activator (genotype AB) have longer progression-free survival and OS than homozygous AA or BB. We could explain these data because both activating and inhibitory KIRs can cause NK cell-mediated lysis, mediated since the NK cell kills tumor cells both when the ligand of an inhibitory KIR is missing and when an activating KIR binds to its ligand. Both circumstances can simultaneously occur in solid tumors treated with cetuximab. Loss of HLA ligands would facilitate cell death mediated by inhibitory KIRs, and the appearance of tumor neoantigens would facilitate tumor lysis by activating KIRs. Therefore, the AB genotype (with sufficient number of inhibitory and activating KIRs) would allow

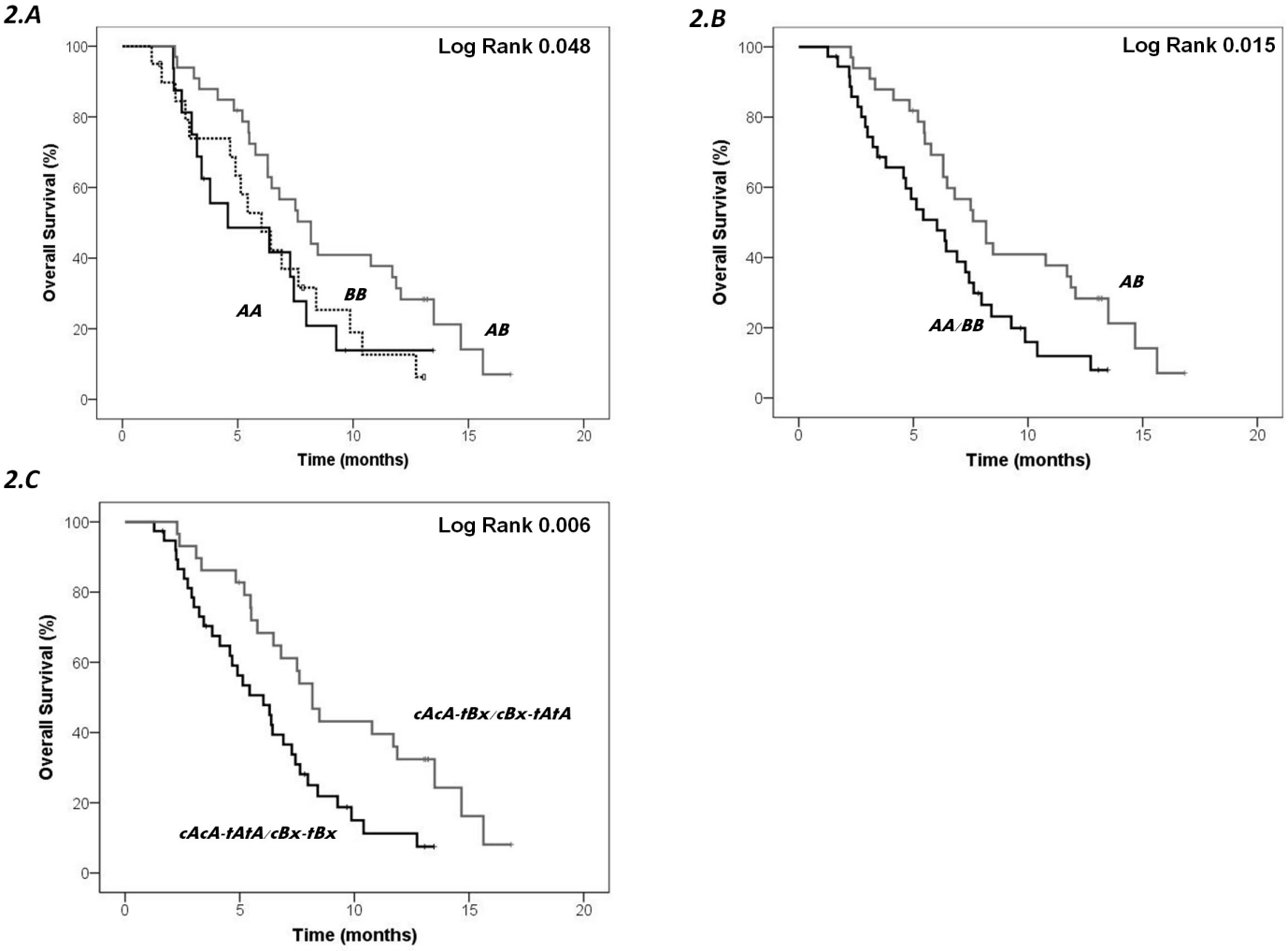

Figure 2 Kaplan-Meier curve for overall survival according to genotypes or centromeric and telomeric KIR content. Heterozygous individuals (AB) were compared with homozygotes (AA or BB) separately (A) or in combination (AA and BB) (B). The median overall survival among homozygotes (AA+BB) was 5.28 months and among heterozygous patients 7.60 months (p=0.015, log-rank test). Genotypes (AA and Bx) were assessed based on the centromeric and telomeric regions (C). The median overall survival among homozygotes (cAcA-tAtA or cBx-tBx) was 5.28 months, whereas among heterozygotes (cAcA-tBx or cBx-tAtA) was 8.17 months (p=0.006, log-rank test). cenA (cA) and telA (tA), centromeric and telomeric motifs of the A haplotype; cenB (cB), centromeric motif B haplotype; KIR, killer cell immunoglobulin-like receptor; telB (tB), telomeric motif of the B haplotype.

patients to be more sensitive to cetuximab, as found in our work where better outcome for patients with AB genotype was observed. Another fact that demonstrates that inhibitory and activating KIRs are interrelated and improve the clinical outcome is the biological interaction between HLA class I ligand and inhibitory KIR that determines NK-cell licensing and subsequent potential to respond to activating signals.[49]

The majority of these studies included patients with bone marrow tumors. Very few have been performed in solid tumors and most of them analyzed cancer risk rather than prognostic impact. Hernandez *et al*[50] showed that Bx genotype, Bx centromere-Bx telomere, cA01|cB03, and tB01|tB01 were associated with risk of developing gastric cancer, and in a different study KIR CEN B haplotype was also associated with increased risk of multiple basal cell carcinoma.[51]

Different authors have tried to demonstrate whether the CEN and TEL semihaplotypes influence the course of treatments differently. Analysis comparing the effect of both halves and scales based on the number of activating semihaplotypes have been performed in different studies.[29] Recently, Ureshino *et al*[52] found an association between different alleles of KIR2DL4, 3DL1 and 2DS4 (present in telA) and treatment response. Other data seem to contradict the previous results and prioritize the role of 2DS1 and 3DS1 (present in telB).[53] The presence of CEN and the absence of TEL KIR B haplotypes were associated with reduced relapse risk of leukemia after (hematopoietic stem-cell transplantation) HSCT for childhood acute lymphoblastic leukaemia (ALL).[54] Cooley *et al*[55] showed the presence of KIR B versus KIR A haplotypes makes better the clinical outcome of patients with acute myelogenous leukemia by decreasing the frequency of

leukemic relapse and enhancing leukemia-free survival. Although CEN and TEL KIR B genes contributed to this effect, the CEN genes were dominant.

In our study, greater progression-free survival and OS were observed in patients with one activating (+) and one inhibitory (−) semihaplotype. Specifically, cAcA-tAtA (−, −) or cBx-tBx (+, +) carriers showed twofold increased risk of progression and death compared with cAcA-tBx (−, +) or cBx-tAtA (+, −) carriers. Again, the advantage of presenting enough number of inhibitory and activating KIRs is observed. This is similar to the study carried out by Mancusi *et al*[53], who observed that donor activating KIRs had no effects on outcomes when donor-versus-recipient NK cell (inhibitory KIR) alloreactivity is lacking. We observed that both the CEN and the TEL semihaplotype appear to have the same power of action, unlike that observed in hematopoietic progenitor transplantation, although in that case the coexistence of KIR and different HLA ligands in donors and recipients may influence the observed results.

No difference was found when patients were classified according to the number of activating semihaplotypes (0–4) or according to the scale of Cooley *et al* (best, better or neutral).[29] These data support the evidence that the presence of both activating and inhibitory KIRs is necessary and that the results are not proportional to the number of activators presented by each subject.

In our work, the role of KIR genotypes in patients with *KRAS* mutant mCRC treated with cetuximab has been evaluated, determining those that are associated with a better clinical outcome. However, our study has some limitations that should be considered. The study has been performed in a limited cohort and therefore should be validated in large-scale prospective studies to confirm whether the biological significance of KIR expression profile could be a fitting prognostic marker for patients with *KRAS* mutant mCRC treated with cetuximab.

CONCLUSIONS

We have demonstrated that a more precise selection of patients with mCRC including KIR genotypes can clearly provide a much higher clinical benefit. From a clinical point of view these new data in this subgroup of patients with *KRAS* mutant mCRC open a therapeutic opportunity to be considered and tested in clinical trials in comparison with other alternatives with scarce benefit.

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Competing interests None declared.

Patient consent for publication Not required.

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