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ORIGINAL ARTICLE

Genetic modulation of the *Let-7* microRNA binding to *KRAS* 3′-untranslated region and survival of metastatic colorectal cancer patients treated with salvage cetuximab–irinotecan

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There is increasing evidence that the *Let-7* microRNA (miRNA) exerts an effect as a tumor suppressor by targeting the *KRAS* mRNA. The *Let-7* complementary site (LCS6) T>G variant in the *KRAS* 3′-untranslated region weakens Let-7 binding. We analyzed whether the LCS6 variant may be clinically relevant to patients with metastatic colorectal cancer (MCRC) treated with anti-epidermal growth factor receptor (EGFR) therapy. LCS6 genotypes and *KRAS*/*BRAF* mutations were determined in the tumor DNA of 134 patients with MCRC who underwent salvage cetuximab–irinotecan therapy. There were 34 G-allele (T/G + G/G) carriers (25%) and 100 T/T genotype carriers (75%). G-allele carriers were significantly more frequent in the *KRAS* mutation group than in patients with *KRAS* wild type ($P = 0.004$). In the 121 patients without *BRAF* V600E mutation, overall survival (OS) and progression-free survival (PFS) times were compared between carriers of the LCS6 G-allele genotypes and carriers of the wild-type T/T genotype. LCS6 G-allele carriers showed worse OS ($P = 0.001$) and PFS ($P = 0.004$) than T/T genotype carriers (confirmed in the multivariate model including the *KRAS* status). In the exploratory analysis of the 55 unresponsive patients with *KRAS* mutation, LCS6 G-allele carriers showed adverse OS and PFS times. These findings deserve additional investigations as they may open novel perspectives for the treatment of patients with MCRC.
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Introduction

MicroRNAs (miRNAs) are a class of small RNAs that have revealed a new level of gene regulation in the cell.[1] After being processed by Drosha and Dicer RNase III endonucleases, mature miRNAs can inhibit the translation of mRNA by directing a RNA-induced silencing complex to the target mRNA.[1] Exerting an effect as either tumor suppressors or oncogenes, miRNAs regulate several genes that have important roles in cancer.[2] Variable levels of miRNAs *in vivo* may affect apoptosis, angiogenesis and specific molecular pathways such as the RAS cascade.[3,4]

Members of the *Let-7* (Lethal-7) family of miRNAs showed RAS regulating activity.[5] *Let-7* induced RAS downregulation after binding to specific sites in the 3′ untranslated region (3′-UTR) of the *KRAS* mRNA.[6,7] These findings led to

growing interest in *Let-7* and its role in cancer development and control.[8–10] It has been recently found that polymorphisms may affect miRNA-mediated regulation of cell functions. They can be present in the 3′-UTR of a target mRNA and in the genes involved in miRNA genesis and maturation.[11] A functional single-nucleotide polymorphism has been described and characterized in the *Let-7* complementary site (LCS) in the *KRAS* 3′-UTR mRNA.[12] The LCS6 single-nucleotide polymorphism (rs61764370) consists in a T-to-G base change and it was found to alter the binding capability of the mature *Let-7* to the *KRAS* mRNA. In experimental models, this variant attenuated the L*et-7* control on *KRAS* with oncogene overexpression.[12] In addition, as a consequence of a possible negative feedback loop, the presence of the LCS6 G variant allele was associated with *Let-7* downregulation. In fact, Chin *et al*.[12] studied four distinct miRNAs of the *Let-7* family and they found that the levels of the *Let-7a, b, d*, and *g* were lower in patients with the variant allele compared with patients without the variant allele.

The frequency of the LCS6 G-allele in Caucasian populations is estimated to be approximately 5–10% in healthy individuals, but it was found to be markedly increased up to 20% in patients with lung cancer.[12] Patients with oral cancer showed adverse survival in the presence of the LCS6 G allele.[13]

The *KRAS* 3′-UTR LCS6 single-nucleotide polymorphism has not been studied in colorectal carcinomas so far. Clinical investigations on this genetic feature may shed light on the influence of the *Let-7* post-transcriptional control on *KRAS in vivo*. In this study we analyzed the *KRAS* 3′-UTR LCS6 variant in the tumor DNA of patients with irinotecan-refractory metastatic colorectal cancer (MCRC) who underwent salvage irinotecan–cetuximab therapy. After excluding cases with *BRAF* V600E mutation,[14] patients were studied for associations between the LCS6 genotypes and overall survival (OS) and progression-free survival (PFS) times. Survival analyses also focused on unresponsive patients whose primary tumors were positive for a *KRAS* mutation.

Materials and methods

The cohort inception was retrospective at four participating institutions in central Italy and the study was approved by the local ethical committees. Patients with irinotecan-refractory MCRC (that is, progressed during or within 3 months after treatment with an irinotecan-based regimen) who underwent salvage cetuximab–irinotecan treatment were included. In addition, patients were required to have histologically confirmed diagnosis of epidermal growth factor receptor (EGFR)-positive adenocarcinoma, measurable disease according to RECIST (response evaluation criteria in solid tumors) criteria and available paraffin-embedded samples of the primary tumor. Pre-treatment evaluation included medical history, clinical–physical examination, ECOG (Eastern Cooperative Oncology Group) performance status evaluation, assessment of metastatic disease based on computed tomography scans, X-ray or other radiographic means, serum chemistries and carcinoembryonic antigen levels. The RECIST criteria were adopted for evaluating response. Patients' characteristics and their outcomes were unknown to investigators performing genetic analyses.

The global population of patients was used to determine the frequency and the distribution of the *KRAS* 3′-UTR LCS6 genotypes according to clinical features and *KRAS*/*BRAF* mutations. After excluding carriers of the *BRAF* V600E mutation,[14] the remaining patients were studied for the association between LCS6 genotypes and OS survival (primary end point). Secondary end points were the analyses of PFS and OS/PFS in patients with unresponsive disease to salvage cetuximab–irinotecan (stable disease or progression) whose tumors were positive for the presence of a *KRAS* mutation.

PFS was defined as the time from the beginning of chemotherapy to first appearance of progression or death by any cause. OS was defined as the time from the beginning of therapy to death or last follow-up.

In Figure 1 the diagram of the flow of the study population is shown.

Genetic analyses

The analyses of *KRAS* 3′-UTR LCS6 genotypes, *KRAS* mutations in codons 12, 13, 61 and the *BRAF* V600E mutation were centralized and performed at the Laboratory of Molecular Biology, Department of Biomolecular Sciences, University of Urbino, Italy.

DNA was extracted from tumor tissue samples using the Qiamp DNA FFPE tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Hotspot mutation sites were amplified by PCR. Primer sequences and conditions are shown in Table 1. Primers design was performed using PSQ Assay Design Software (Biotage, Uppsala, Sweden).

Each PCR reaction contained 50–150 ng of DNA, 0.4 μM of each primer, 12.5 μl of PCR Master Mix (Diatheva, Fano, Italy) and 0.625 U of HotStarTaq polymerase (Diatheva) in a total volume of 25 μl. Successful and specific amplification of the region of interest was verified by visualizing 5 μl of the PCR product on a 2% agarose gel. Preparation of the single-stranded DNA template for pyrosequencing analysis was performed using the PSQ Vacuum Prep Tool (Biotage) according to the manufacturer's instructions. A total of 20 μl of biotinylated PCR product was immobilized on streptavidin-coated Sepharose High-Performance beads (Amersham Biosciences, Piscataway, NJ, USA) and processed to obtain a single-stranded DNA using the PSQ 96 Sample Preparation Kit (Biotage) according to the manufacturer's instructions.

The template was incubated with 0.4 μmol sequencing primer at 80 °C for 2 min in a PSQ96 plate. The sequencing by synthesis reaction of the complementary strand was automatically performed on a PSQ 96MA instrument (Biotage) using PyroGold reagents (Biotage).

Statistical analysis

The distribution of genotypes was tested for Hardy–Weinberg equilibrium (Figure 1). According to the functional data on the *KRAS* 3′-UTR LCS6 G-allele and the expected low frequency of the homozygous variant genotype, it was planned to collapse in the analyses the G/T and the G/G genotypes (G-allele genotypes).

Two-tailed Fisher's exact test was used to compare proportions between carriers of the wild-type T/T genotype and carriers of the G allele genotypes (G/T + G/G). OS and PFS times were estimated using the Kaplan–Meier method. The associations between genotypes and survival times were tested using the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using the multivariate Cox's proportional hazard regression model, with adjustment for the presence of *KRAS* mutation (mutation present versus wild-type status), sex (male versus female) age (continuous variable), ECOG performance status (0 versus 1), number of metastatic sites (1 versus $\geqslant 2$) and carcinoembryonic antigen levels (continuous variable).

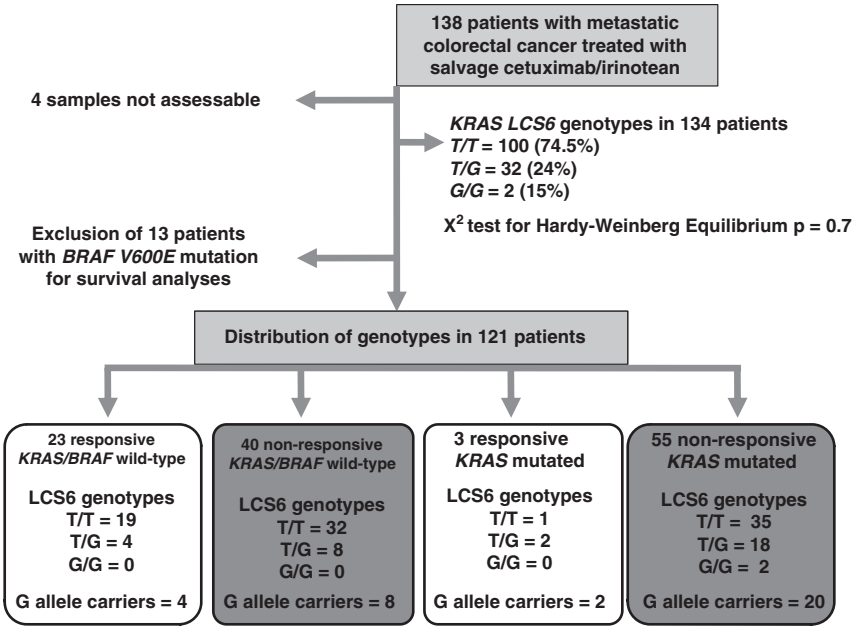

Figure 1 Diagram showing the flow of the patients in the study. All assessable cases for genotyping were studied for evaluating the frequency of the *KRAS* 3′-UTR LCS6 genotypes. After excluding carriers of the *BRAF V600E* mutation, survival analyses were performed in 121 patients with and without *KRAS* mutations.

Table 1 Primers and conditions for the analyses

Mutations	*Primers*	*PCR conditions*
KRAS codons 12–13	F: 5′-GGCCTGCTGAAAATGACTGAA-3′ R: 5′-[Btn]-TTCGTCCACAAAATGATTCTGA-3′ Seq: 5′-TATAAACTTGTGGTAGTTGG-3′ Dispensation order: 5′-CTAGCATACTGATCGTAGC-3′	10′ at 95 °C, 40 cycles of 15″ at 95 °C, 30″at 64 °C, 40″ at 72 °C and 5′ at 72 °C
KRAS codon 61	F: 5′-CAGACTGTGTTTCTCCCTTCTCA-3′ R: 5′-[Btn]CTCATGTACTGGTCCCTCATTG-3′ Seq: 5′-ATATTCTCGACACAGCAG-3′ Dispensation order: 5′-GTCGATCGATCGATGAG-3′	10′ at 95 °C, 40 cycles of 15″ at 95 °C, 30″ at 66 °C, 30″ at 72 °C and 5′ at 72 °C
BRAF codon 600	F: 5′-ATGCTTGCTCTGATAGGAA-3′ R: 5′-[Btn]-GCATCTCAGGGCCAAA-3′ Seq: 5′-GGTGATTTTGGTCTAGCTAC-3′ Dispensation order: 5′-CAGTACGATCT-3′	10′ at 95 °C, 40 cycles of 15″ at 95 °C, 30″ at 54 °C, 40″ at 72 °C and 5′ at 72 °C
KRAS 3′-UTR LCS6 T/G	F: 5′ -TTTTAGGAGAGACGGGGTTTCA-3′ R: 5′-[Btn]-TGAGTTCTGCAAAACAGGTTTATG-3′ Seq: 5′-TCCTGACCTCAAGTGAT-3′ Dispensation order: 5′-CGTACAC-3′	10′ at 95 °C, 40 cycles of 15″ at 95 °C, 30″ at 63 °C, 30″ at 72 °C and 5′ at 72 °C

Abbreviations: Btn, biotynilated; Seq, sequencing primer; 3′-UTR, 3′ untranslated region.

To further explore the possible influence of the LCS6 variant, an analysis for estimating and comparing PFS and OS times in unresponsive patients with *KRAS* mutations was planned.

In all analyses, statistical significance was set at two-sided $P<0.05$ value.

Results

Characteristics of patients

In 134 patients there were 100 carriers of the wild-type LCS6 T/T genotype (75%) and 34 carriers of the LCS6 G variant allele (T/G and G/G) genotypes (25%). *KRAS* mutations in codons 12, 13 and 61 were found in 58 patients (43%) and the *BRAF* V600E mutation occurred in 13 patients (10%). All patients received third-line therapy with cetuximab plus irinotecan according to the schedule commonly used in clinical practice: cetuximab 250 mg m^{-2} intravenously, day 1 weekly (loading dose: 400 mg m^{-2} intravenously, day 1 in the first cycle) and irinotecan 180 mg m^{-2} intravenously, day 1 every 2 weeks. The characteristics of the 134 studied patients are shown in Table 2. No significant differences between carriers of the LCS6 wild-type T/T genotype and carriers of the G variant (T/G and G/G genotypes) were found.

LCS6 genotypes and KRAS/BRAF status

As shown in Table 3, the distribution of carriers of the LCS6 genotypes was significantly different among carriers of *KRAS* and *BRAF* mutations. In particular, all the 13 *BRAF* V600E mutation carriers were LCS6 T/T wild type and the majority of LCS6 G-allele carriers were in the *KRAS* mutation group.

Table 2 Characteristics of the 134 patients

Median age	
Years (range)	65 (41–77)
Sex (n, %)	
Male	73 (54)
Female	61 (46)
ECOG performance status (n, %)	
0	65 (48)
1–2	69 (52)
No. of metastatic sites (n, %)	
1	32 (24)
2	61 (45)
>2	41 (31)
Pattern of metastatic disease (n, %)	
Liver involvement	106 (79)
Peritoneal carcinomatosis	31 (23)
Non-liver–non-carcinomatosis	17 (13)
Carcinoembryonic antigen (n, %)	
<10 ng ml^{-1}	44 (33)
>10 ng ml^{-1}	90 (67)

Abbreviation: ECOG, Eastern Cooperative Oncology Group.
None of the characteristics showed significant association with LCS6 genotypes.

Survival analyses

There were 121 assessable patients without the *BRAF* V600E mutation (Figure 1). In this study population, PFS and OS were 3.5 months (6 censored observations) and 10.6 months (28 censored observations), respectively. As shown in Figure 2, the log-rank comparison of OS (Figure 2a) and PFS (Figure 2b) curves between LCS6 T/T genotype carriers and G allele carriers found significant differences in terms of adverse outcomes for the presence of the variant allele. In the planned multivariate model, LCS6 G allele (HR 1.68; 95% CI 1.14–2.7; $P=0.002$), worse ECOG performance status (HR 1.57; 95% CI 1.01–2.5; $P=0.04$) and *KRAS* mutation (HR 2,3; 95% CI 1.46–3.7; $P=0.004$) were significantly associated with adverse OS. LCS6 G allele (HR 1.59; 95% CI 1.04–2.75; $P=0.03$) and *KRAS* mutation (HR 2.0; 95% CI 1.34–2.9; $P=0.006$) were significantly associated with adverse PFS. Notably, there was a different distribution of LCS6 genotypes between patients with disease control (patients with complete response, partial response and stable disease) and patients with disease progression (Table 4).

In the exploratory analysis in 55 unresponsive patients with *KRAS* mutation, G-allele carriers and T/T carriers showed median OS of 5.9 and 9.7 months (HR 1.77; 95% CI 1.02–3.8) and median PFS of 2.5 and 3.4 months (HR 1.78; 95% CI 1.1–4.14), respectively. The log-rank comparison of OS (Figure 3a) and PFS (Figure 3b) curves showed significant differences in terms of adverse outcomes for the presence of the variant allele.

The G-allele could also have a role in patients with *KRAS*/*BRAF* wild-type status and its presence could represent an unfavorable predictive marker to the anti-EGFR therapy. However, there were 12 G-allele carriers only among the 63 patients with *KRAS*/*BRAF* wild-type tumors. With this limitation, the log-rank comparison of PFS and OS times showed a trend for unfavorable outcomes in G allele carriers, but the difference was not significant. G allele and T/T carriers showed median OS of 9 and 14.2 months (HR 1.9; 95% CI 0.98–5.93) and median PFS of 3.7 and 5.3 months (HR 1.45; 95% CI 0.73–3.35), respectively.

Discussion

In the past years, anti-EGFR therapy with monoclonal antibodies, cetuximab and panitumumab, has represented a major improvement in the treatment of patients with MCRC. Unfortunately, almost all patients with *KRAS* mutation do not respond to anti-EGFR therapy[15] and the baseline determination of the *KRAS* status has become mandatory for treating patients with MCRC.[16] To date, there is no alternative therapy for patients with *KRAS* mutation who are excluded from anti-EGFR therapy and for those who do not respond even in the presence of the *KRAS* wild-type status. With this in mind, we planned an investigation

Table 3 Distribution of the *KRAS 3′-UTR* LCS6 genotypes according to *KRAS* and *BRAF* mutational status

	Overall population (134 patients) N (%)	*LCS6 genotypes*		
		T/T (100 patients) N (%)	*G/T+G/G (34 patients) N (%)*	P-*value*
KRAS				
Wild type	76 (57)	64 (64)	12 (35)	0.004
Mutation present	58 (43)	36 (36)	22 (65)	
BRAF V600E				
Wild type	121 (90)	87 (87)	34 (100)	0.03
Mutation present	13 (10)	13 (13)	0 (0)	

Abbreviation: 3′-UTR, 3′ untranslated region.

Table 4 Tumor response and disease control rate according to the *KRAS* 3′-UTR LCS6 genotypes in 121 assessable patients

	N (%)	*LCS6 genotypes*		
		T/T (87 patients) N (%)	*G/T+G/G (34 patients) N (%)*	P-*value*
Responders	26 (21)	20 (23)	6 (18)	0.6
Non-responders	95 (79)	67 (77)	28 (82)	
Disease control rate[a]	71 (59)	57 (66)	14 (41)	0.02
Progressions	50 (41)	30 (34)	20 (59)	

Abbreviation: 3′-UTR, 3′ untranslated region.
[a]Patients with complete response, partial response and stable disease were included in the disease control rate group.

for evaluating a genetic feature, which was found to modulate the tumor suppressor function of the *Let-7* miRNA on the *KRAS* pathway.

The first finding in the global study population of MCRC patients was the relatively high prevalence of the variant LCS6 G-allele with a combined frequency of the heterozygous T/G and the homozygous G/G genotypes of 25%. Chin *et al.*[12] found a 20% prevalence of the variant allele in non-small cell lung cancer patients. According to existing databases of genetic variations,[17] the frequency of the variant G-allele is variable across population, achieving in European population the highest prevalence of approximately 10%. Chin *et al.*[12] found a possible association between the LCS6 variant allele and the risk of non-small cell lung cancer and it is likely that the same association may exist in colorectal cancer. We did not study the frequency of the LCS6 genotypes in healthy individuals as this kind of analysis was beyond the scope of our investigation. However, in light of these data, a case–control study that focuses on the possible role of the LCS6 variant on the risk of colorectal cancer is warranted.

The second finding was the particular distribution of the LCS6 genotypes according to the *KRAS/BRAF* mutational status. The only available study published on LCS6 was performed in non-small cell lung cancer patients,[12] and given the low frequency of *KRAS* mutations in this tumor, the researchers could not analyze the relationship between the *KRAS* 3′-UTR LCS6 genotypes and the *KRAS* status. We found a prevalence of the LCS6 G-allele genotypes in *KRAS* mutated cases and their absence in carriers of *BRAF* V600E mutation. If these associations will be confirmed in additional series, the underlying mechanisms will be worthy of investigations and additional studies in normal tissues will be also required for *KRAS* quantification in relation to the LCS6 variant. At the moment, we could hypothesize that some clonal selection may occur in the tumor and it favors less differentiated and more aggressive clones that harbor both *KRAS* mutation and the *KRAS* 3′-UTR LCS6 G-allele variant.[18,19] In addition, an intriguing hypothesis is that the *KRAS* 3′-UTR LCS6 G-allele promotes some mechanisms in the colorectal cancer microenvironment that facilitate the development of *KRAS* mutations.

The primary end point of the study, which was the clinical influence of the genetic modulation of the *Let-7* binding, supports further development of the miRNA in therapeutics. In fact, we found that the presence of the *KRAS* 3′-UTR LCS6 G-allele was associated with adverse survival outcomes in MCRC patients treated with salvage cetuximab–irinotecan therapy. A number of experimental investigations have shown the tumor growth suppression effect of the *Let-7*

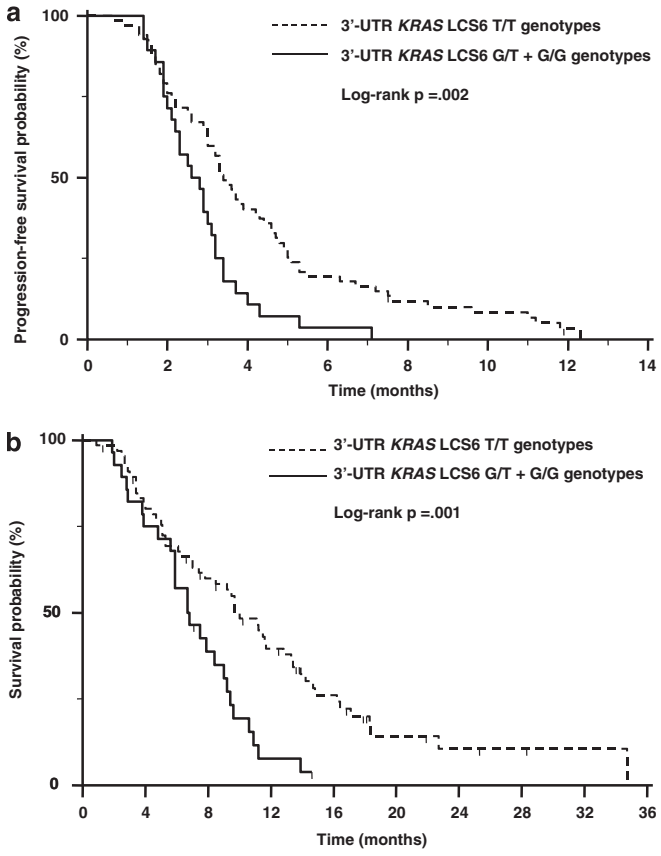

Figure 2 Analysis of overall survival (**a**) and progression-free survival (**b**) curves in 121 patients with comparison between carriers of the *KRAS* 3′-UTR LCS6 G-allele genotypes (G/T + G/G) and carriers of the wild-type T/T genotype.

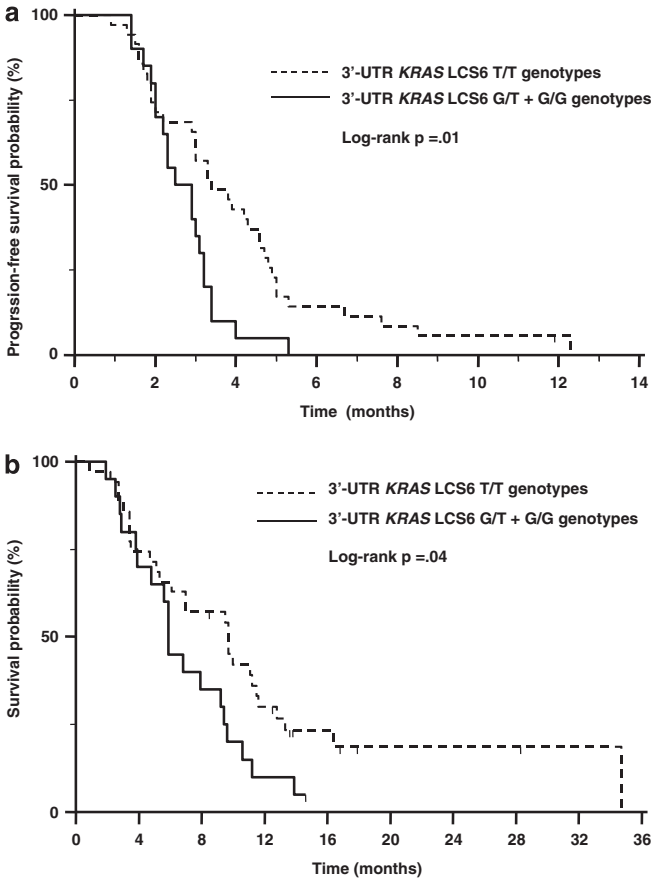

Figure 3 Analysis of overall survival (**a**) and progression-free survival (**b**) curves in the 55 unresponsive patients with *KRAS* mutation with comparison between carriers of the *KRAS* 3′-UTR LCS6 G-allele genotypes (G/T + G/G) and carriers of the wild-type T/T genotype.

miRNA.[8–10,20–22] Therefore, it is not surprising that *in vivo*, *Let-7* and *Let-7* regulators may show an effect on clinical outcomes as recently found in patients with oral[13] and lung cancer.[23] Notably, the influence of the LCS6 variant on PFS and OS was present in the 55 unresponsive patients who were carriers of a *KRAS* mutation.

In general, the worse survival times in G-allele carriers than in wild-type T/T genotype carriers would suggest that preserved *Let-7* function may exert some control on the RAS pathway with additive effect to the anti-EGFR blockade. If the anti-EGFR blockade does not work, because of the presence of an activating *KRAS* mutation, the downstream control of *Let-7* may still ensure some *KRAS* downregulation, provided that there is preserved binding between the miRNA and the mRNA of the target gene. If confirmed in additional series, these findings could also apply to a hypothetical study on *Let-7*-like interference for downregulating *KRAS,* in which the clinical activity could depend on the absence of the unfavorable LCS6 G-allele.

In conclusion, further studies are warranted in this field as they may open novel perspectives for the treatment of patients with advanced MCRC and for those who are unresponsive to the anti-EGFR therapy in particular.[24,25] In this perspective, there is the possibility that more than one miRNA is implicated and exploited in the control of the RAS pathway.[26,27] Future studies should also analyze the effect of *Let-7* and its genetic modulation in patients with early disease and in patients treated with chemotherapy.[28,29]

Conflict of interest

The authors declare no conflict of interest.

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