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A *Let-7* MicroRNA SNP in the *KRAS* 3'UTR Is Prognostic in Early-Stage Colorectal Cancer

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Abstract

Purpose: Colorectal cancer (CRC) is a common cause of death worldwide. Tumor-node-metastasis-system stage is currently used to guide therapy decisions but lacks precision. Prognostic biomarkers are needed to refine stratification of patients for chemotherapy but validated biomarkers are not yet available. Recently, a SNP in a *lethal-7* (*let-7*) miRNA complementary site (LCS6) in the *KRAS* 3'untranslated region was suggested to affect survival in metastatic CRC. Effects in early-stage CRC are however unknown. We studied *KRAS-LCS6* genotype, hypothesizing that it might identify early-stage cases with a poor prognosis, and could potentially be used in therapy decision-making.

Experimental Design: We studied 409 early stage, 182 stage III, and 69 stage IV cases, and 1,886 subcohort members from the Netherlands Cohort Study. *KRAS-LCS6* genotype was assessed with TaqMan PCR. Kaplan–Meier analyses or Cox regression were used to assess associations between genotype and CRC risk or cause-specific survival.

Results: Early-stage cases with the *KRAS-LCS6* variant had a lower CRC risk (incidence-rate ratio 0.68; 95% CI: 0.49–0.94) and a better survival (log-rank $P = 0.038$; HR 0.46; 95% CI: 0.18–1.14). In patients with *KRAS*-mutated CRC carrying the *KRAS-LCS6* variant, the better outcome was enhanced as no patients died of CRC (log-rank $P = 0.017$). In advanced patients, no clear association between genotype and CRC risk or survival was observed.

Conclusions: Our results indicate that early-stage CRC cases with the *KRAS-LCS6* variant have a better outcome. In advanced disease, the better outcome no longer exists. For early-stage patients, *KRAS-LCS6* genotype combined with *KRAS* mutations merits validation as a prognostic biomarker and consideration in therapy decision-making. *Clin Cancer Res*; 17(24); 7723–31. ©2011 AACR.

Introduction

Despite diagnostic and therapeutic innovations, colorectal cancer (CRC) remains the second cause of cancer death in the western world (1). The tumor-node-metastasis-system (TNM) is currently the main tool to provide prognostic information; it is highly predictive for prognosis at the extremes, but less predictive for intermediate stages (2, 3). According to current guidelines, adjuvant chemotherapy is

not given to early-stage patients (T1-3-N0-M0 according to the International Union Against Cancer-TNM) as 5-year survival rates in this group are more than 70%. Nevertheless, 20% to 30% of early-stage patients (stage I and II) will die of CRC within 5 years, evoking the question whether these deaths could have been avoided if these patients were identified in advance and therapy was adapted accordingly. Previously, numerous studies have been published claiming a prognostic influence of molecular markers. Results however, are inconsistent and the question which molecular alterations influence prognosis remains unresolved (4).

Over the last years, a new class of gene regulators, microRNAs (miRNA), has been identified as important factors in cancer development and progression. Evidence suggests that one miRNA can regulate many mRNAs simultaneously (5) and miRNAs can act as both tumor suppressors and oncogenes (6). One of the first discovered miRNA families is the *lethal-7* (*let-7*) family of miRNAs, and altered expression of these miRNAs has been described in many cancers (7). In lung cancer, *let-7* is poorly expressed (8, 9), overexpression of *let-7* inhibits cell growth *in vitro* (9) and *in vivo* (10, 11) suggesting that *let-7* miRNAs may act as tumor suppressors (6). In colon cancer cells, *let-7* expression was significantly decreased in tumor tissue as compared with adjacent

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J.B. Weidhaas and M. van Engeland contributed equally to this work.

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Translational Relevance

We report for the first time that a SNP in a *let-7* (*let-7*) complementary site (LCS) in the *KRAS* 3'-UTR (*KRAS-LCS6*) might be a prognostic biomarker in early-stage colorectal cancer (CRC). The *KRAS-LCS6* variant is known to cause higher levels of the *KRAS* oncogenic protein and lower levels of the tumor suppressor *let-7* miRNAs. We studied the influence of *KRAS-LCS6* in 409 early-stage (stage I and II), 182 stage III, and 69 stage IV cases from the large, prospective Netherlands Cohort Study (NLCS). Early-stage patients with the *KRAS-LCS6* variant had a better prognosis, especially those that also had *KRAS* mutations, and this was independent of microsatellite instability or other prognostic factors. In addition, we studied the influence of the *KRAS-LCS6* variant on CRC risk using data from 1,886 subcohort members from the NLCS. The G-allele was associated with a decreased risk on early-stage CRC, but was not associated with advanced stage CRC risk, suggesting that the G-allele is not associated with the likelihood of advanced stage CRC. As our population is the only untreated population studied to date, our results give a first insight into the natural biology of CRC with the *KRAS-LCS6* variant. The *KRAS-LCS6* variant may become a new biomarker in CRC to guide treatment decisions in early-stage patients.

noncancerous tissue (12). In addition, *let-7* expression was increased and RAS expression was decreased in cell lines after transfection of a *let-7a-1* miRNA precursor suggesting that *let-7* is involved in regulating colon cancer cell growth (12). miRNAs usually control gene expression by binding to complementary elements in the 3'untranslated region (UTR) of target mRNAs (6). It has been shown that *let-7* induces RAS downregulation after binding to specific sites in the 3'-UTR *KRAS* mRNA (6, 7). Recently, a SNP in a *let-7* complementary site (LCS) in the *KRAS* 3'-UTR (*KRAS-LCS6*) has been identified that affects *let-7*-mediated regulation of *KRAS* expression. The variant G-allele was observed to lead to higher *KRAS* levels and lower *let-7* levels as compared with the wild type (13). G-allele carriers have been shown to have an increased lung cancer risk in moderate smokers (13), an increased ovarian cancer risk (14) although perhaps not for all women (15), an increased triple-negative breast cancer risk (16), a reduced survival in oral cancers (17) but not in lung cancer (18). Recently, in *KRAS/BRAF*-mutated CRC, G-allele carriers showed a reduced survival in late-stage CRC (19) and an altered response to cetuximab (19, 20), supporting the function of this variant in colon cancer. As the influence of *KRAS-LCS6* genotype in early-stage CRC has not been determined, we assessed the influence on prognosis in 409 early-stage (TNM stage I and II; T1-4, N0, M0), 182 stage III (T1-4, N1, M0), and 69 stage IV (T1-4, N0-1, M1) CRC cases from a large prospective cohort study. We assessed the influence of

KRAS-LCS6 genotype on CRC risk using data from 1,886 subcohort members from the Netherlands Cohort Study on diet and cancer (NLCS).

Materials and Methods

Study population

Until 1994, 925 incident CRC cases (ICD-O: 153.0–154.1) were identified within the NLCS which started in 1986 with 120,852 healthy persons between 55 and 69 years. Incident cancer cases were identified by linkage with the Netherlands Cancer Registry (NCR) and PALGA, a nationwide registry of histopathology and cytopathology (21). The NLCS has been described in detail elsewhere (22). A total of 815 patients could be linked to PALGA and paraffin-embedded tumor tissue was collected from 54 pathology registries throughout the Netherlands. We were able to extract sufficient, good quality DNA for 734 (90%) cases (23). At baseline, a subcohort of 5,000 healthy persons was randomly sampled from the entire cohort to estimate person-years at risk of the cohort through biennial follow-up of vital status. For 1,886 persons, DNA from buccal swabs was available for *KRAS-LCS6* genotyping.

Data collection

Information on tumor localization, stage, differentiation grade, incidence date, and treatment in the 3 months after diagnosis, was available through the NCR. Vital status until May 2005 was retrieved from the Central Bureau of Genealogy and the municipal population registries and could be obtained for all 734 cases. Causes of death were retrieved through linkage with Statistics Netherlands. CRC-related deaths were defined as deaths as a result of a carcinoma in the colon, rectosigmoid, rectum, gastro-intestinal tract (nonspecific) or liver metastases. In the case of gastro-intestinal (nonspecified) or liver metastases, we used the information from NCR and PALGA to eliminate the possibility of another primary cancer as cause of death.

DNA isolation and *KRAS-LCS6* SNP determination

A 5- μ m section of each tumor tissue block was stained with haematoxylin and eosin and revised by a pathologist. Five sections of 20 μ m were deparaffinated and DNA was extracted using the Puregene DNA isolation kit (Gentra systems) according to the manufacturer's instructions. In brief, cell lysis solution and proteinase K (20 mg/mL, Qiagen) were added to the tissue and incubated overnight at 55°C. DNA was extracted for 72 hours at 37°C, protein was removed, and DNA was precipitated using 100% 2-propanol. Finally, DNA was rehydrated in hydration buffer. Isolated DNA was amplified using TaqMan PCR assays designed specifically to identify the T or G allele of the *KRAS-LCS6* SNP (Applied Biosciences). Although we used tumor DNA to assess genotype, it has previously been well documented that the genotype of normal and tumor tissue is the same in *KRAS-LCS6* variant allele carriers (13).

KRAS and *BRAF* mutations were assessed by nested PCR and direct sequencing (*KRAS*), and restriction fragment

length polymorphism (*BRAF*) as described previously (23, 24). Promoter methylation of *RASSF1A*, *O⁶-MGMT*, *CHFR*, and *CIMP* markers as proposed by Weisenberger (25) was assessed by chemical modification of genomic DNA with sodium bisulfite and methylation-specific PCR (MSP; refs. 24, 26, 27). Microsatellite instability (MSI) status was determined using BAT-26, BAT-25, NR-21, NR-22, and NR-24 as described previously (28). All assays were done and analyzed blinded to the main study endpoint, that is, CRC-related death.

Statistical analyses

Cause-specific survival was defined as time from cancer diagnosis until CRC-related death or end of follow-up. Kaplan–Meier curves and log-rank tests were used to estimate the influence of the *KRAS* variant on cause-specific survival. HR and corresponding 95% CI were assessed by use of Cox proportional hazard models adjusted for potential confounders. Factors were considered possible confounders if they were known prognostic factors for CRC and influenced the crude HR by more than 10%. Confounders that were included were age at diagnosis (continuous), sex, tumor differentiation grade (well, moderate, poor, and undifferentiated), and location (proximal, distal, rectosigmoid, and rectum). The proportional hazard assumption was tested using the Schoenfeld residuals and the log(–log) hazards plots. Survival analyses were restricted to 10 years after diagnosis as CRC-related cause of death was unlikely after that point. Incidence rate ratios (RR) and 95% CI were estimated using Cox proportional hazards models. Standard errors were estimated using the robust Huber–White sandwich estimator to account for additional variance introduced by sampling from the cohort. All analyses were done with the statistical package STATA10.0.

Results

CRC variables and the *KRAS-LCS6* variant

Patients in this study were more often male (55.6%), diagnosed with an early-stage tumor (62.0%) or a proximal or distal tumor (65.3%; Table 1). During follow-up, 41.4% of the patients died of CRC. The *KRAS-LCS6* variant was detected in 14.0% of early-stage (stage I and II), in 19.2% of stage III and 21.4% of stage IV patients ($P = 0.160$; $P_{\text{trend}} = 0.060$). *KRAS-LCS6* variant patients were more often diagnosed with advanced stage disease (47.5% vs. 36.9% in wild-type patients, $P = 0.046$). No other statistically significant differences were found between wild type and variant carriers for sex, age at diagnosis, differentiation grade, tumor location, MSI, or mutations in *KRAS* (Table 1), *BRAF* ($P = 0.640$), or *RASSF1A* promoter CpG island methylation ($P = 0.423$). As expected, patients with stage III or IV disease more often died from CRC ($P < 0.001$) and more often had a poorly differentiated tumor ($P < 0.001$). Advanced stage patients more often had a proximal ($P = 0.036$) or MSS tumor ($P = 0.047$) as compared with early-stage patients.

Stage IV G-allele *KRAS-LCS6* carriers were more likely to be female (66.7%; $P = 0.097$), and to present with a

proximal tumor (71.4%; $P = 0.004$) as compared with G-allele carriers in other stages (Table 2).

The *KRAS-LCS6* variant is associated with better survival in early-stage CRC

No statistically significant difference was observed in Kaplan–Meier analyses for the *KRAS-LCS6* variant and cause-specific survival in the total population (log-rank test, $P = 0.864$) (Supplementary Fig. S1).

As survival depends on cancer stage, we conducted analyses stratified for stage. Early-stage G-allele carriers showed a statistically significantly better survival as compared with wild-type cases (log-rank test, $P = 0.038$; Fig. 1A). This difference was not seen for advanced stage cases (Fig. 1B and C; log rank, $P = 0.775$ and 0.875 for stage III and IV cases, respectively).

KRAS/BRAF mutation status enhances the association between the *KRAS-LCS6* variant and survival

Figure 2A shows Kaplan–Meier analyses for early-stage (stage I and II) CRC cases with the *KRAS-LCS6* variant and *KRAS* mutations. In our population, none of the 20 G-allele carriers with *KRAS* mutations died due to CRC. *KRAS-LCS6* wild-type patients had a poorer survival, especially if they had *KRAS* mutations (log-rank test, $P = 0.043$; log-rank test *KRAS-LCS6* G-allele carriers with *KRAS* mutations compared with *KRAS-LCS6* G-allele carriers without *KRAS* mutations $P = 0.017$). This observation was independent of T stage; among 115 *KRAS-LCS6* wild-type cases with *KRAS* mutations, only 5 (4%) were diagnosed as high-risk stage IIb (T4N0M0). Among G-allele carriers, no patients were diagnosed as stage IIb. For advanced stage patients, no survival difference was seen (Fig. 2B and C, log-rank test, $P = 0.535$ for stage III and $P = 0.989$ for stage IV)) although results for stage III patients suggest that *KRAS-LCS6* wild-type patients with *KRAS* mutations have the worst prognosis. Subgroup analysis showed that the better outcome for early-stage *KRAS-LCS6* variant carriers was mainly caused by stage II cases. Analyses stratified for T stage were not possible due to limited patient numbers (data not shown).

BRAF mutated CRCs carrying the G-allele showed a similar better outcome, although this was not statistically significant (log-rank test, $P = 0.166$) possibly due to small number of patients carrying both events (9 patients). Similarly, G-allele carriers with aberrant *RASSF1A* promoter hypermethylation, another gene involved in the Ras pathway, had a better prognosis, although only borderline statistically significant, as compared with wild-type carriers without *RASSF1A* hypermethylation (log-rank test, $P = 0.062$). Analyses combining *KRAS*, *BRAF*, and *RASSF1A* status showed that early-stage G-allele carriers with additional alterations in *KRAS*, *BRAF*, or *RASSF1A* have a better prognosis (log-rank test, $P = 0.026$). In contrast, when adding methylation status of genes not involved in the Ras pathway such as *MGMT* or *CHFR*, no survival differences were observed (*MGMT*: log-rank test, $P = 0.220$; *CHFR*: log-rank test, $P = 0.118$; data not shown).

Table 1. Baseline characteristics for the total population, KRAS-LCS6 variant and wild-type carriers and early-stage and advanced stage CRC cases within the NLCS on diet and cancer, 1986–1994

	Overall	KRAS-LCS6 wild-type TT	KRAS-LCS6 variant G-allele (He+Ho)	P	Early-stage (stage I and II) CRC	Stage III	Stage IV	P
Total population, n (%)	734 (100)	567 (83.6)	111 (16.4)		409 (62.0)	182 (27.6)	69 (10.5)	
Sex [male, n (%)]	406 (55.6)	308 (54.3)	66 (59.5)	0.320	219(53.6)	102 (56.0)	33(47.8)	0.506
Age at diagnosis (mean, SD)	67.9 (4.3)	67.9 (4.3)	67.9 (4.4)	0.885	68.0 (4.4)	67.5 (4.1)	68.5 (3.8)	0.203
CRC-related death (yes, n (%))	302 (41.4)	230 (40.6)	46 (42.2)	0.761	95 (23.3)	107 (8.8)	66(95.6)	<0.001
Cancer stage, n (%)	409 (62.0)	326 (63.1)	53 (52.5)					
III	182 (27.6)	137 (26.5)	33 (32.7)					
IV	69 (10.5)	54 (10.4)	15 (14.9)	0.124				
Differentiation, n (%)	74 (11.5)	58 (11.8)	9 (8.7)		46 (12.7)	13 (7.8)	3 (5.0)	
Well	457 (71.0)	354 (71.8)	72 (69.9)		277(76.5)	109 (65.3)	36 (60.0)	
Moderate	106 (16.5)	75 (15.2)	21 (20.4)		37 (10.2)	41 (24.6)	20 (33.3)	
Poor	7 (1.1)	6 (1.2)	1 (1.0)	0.532	2 (0.6)	4 (2.4)	1 (1.7)	<0.001
Undifferentiated	239 (33.2)	196 (35.4)	34 (31.2)		128 (31.5)	63 (34.8)	33 (49.3)	
Location, n (%)	231 (32.1)	177 (32.0)	37 (33.9)		125 (30.7)	61 (33.7)	22 (32.8)	
Proximal	80 (11.1)	59 (10.6)	11 (10.1)		53 (13.0)	17 (9.4)	5 (7.5)	
Distal	169 (23.5)	122 (22.0)	27 (24.8)	0.824	101 (24.8)	40 (22.1)	7 (10.5)	0.036
Rectosigmoid	578 (87.3)	463 (87.5)	88 (84.6)		314 (84.9)	149 (88.7)	63 (95.5)	
Rectum	84 (12.7)	66 (12.5)	16 (15.4)	0.420	56 (15.1)	19 (11.3)	3 (4.5)	0.047
Molecular characteristics, n (%)	167 (27.7)	127 (24.5)	34 (35.4)					
MSS	436 (72.3)	352 (73.5)	62 (64.6)	0.076				0.121
MSI	464 (63.2)	362 (63.8)	69 (62.2)		263 (64.3)	121 (66.5)	39 (56.5)	
CIMP+	270 (36.8)	205 (36.2)	42 (37.8)	0.736	146 (35.7)	61 (33.5)	30 (43.5)	0.336
CIMP-	567 (83.6)	463 (87.5)	88 (84.6)		326 (86.0)	137 (80.6)	54 (78.3)	
KRAS mutations, n (%)	107 (15.8)	4 (0.6)	2 (0.5)		51 (13.5)	32 (18.8)	15 (21.7)	
Wild type								
KRAS variant								
Variant He								
Variant Ho								
								0.298

Table 2. Baseline and molecular characteristics for early stage, stage III and IV patients according to KRAS-LCS6 status

		KRAS-LCS6 wild-type TT				KRAS-LCS6 variant G-allele (He+Ho)			
		Stage I and II	Stage III	Stage IV	P	Stage I and II	Stage III	Stage IV	P
Total population,		326 (63.1)	137 (26.5)	54 (10.4)		53 (52.5)	33 (32.7)	15 (14.9)	
	n (%)								
T1		43 (13.2)	1 (0.7)	–		8 (15.1)	–	–	
T2		95 (29.1)	13 (9.6)	3 (5.6)		17 (32.1)	5 (15.6)	–	
T3		174 (53.4)	113 (83.1)	41 (75.9)		27 (50.9)	24 (75.0)	12 (80.0)	
T4		14 (4.3)	9 (6.6)	10 (18.5)	<0.001	1 (1.9)	3 (9.4)	3 (20.0)	0.001
Sex [male, n (%)	Male	173 (53.1)	75 (54.7)	28 (51.9)	0.920	29 (54.7)	22 (66.7)	5 (33.3)	0.097
Age at diagnosis		68.0 (0.3)	67.5 (0.4)	68.5 (0.5)	0.283	68.0 (0.6)	67.5 (0.7)	68.5 (1.2)	0.756
(mean, SD)									
CRC-related	Yes	79 (24.2)	79 (57.7)	51 (96.2)	<0.001	8 (15.7)	19 (57.6)	14 (93.3)	<0.001
death [yes,									
n (%)									
Differentiation,	Well	38 (13.2)	9 (7.3)	2 (4.4)		5 (10.6)	2 (6.3)	1 (7.1)	
n (%)									
	Moderate	224 (77.8)	77 (62.1)	31 (67.4)		35 (74.5)	24 (75.0)	5 (35.7)	
	Poor	25 (8.7)	34 (27.4)	12 (26.1)		6 (12.8)	6 (18.8)	8 (57.1)	
	Undifferentiated	1 (0.4)	4 (3.2)	1 (2.2)	<0.001	1 (2.1)	–	–	0.028
Location, n (%)	Proximal	107 (33.0)	55 (40.2)	23 (43.4)		15 (28.3)	6 (18.8)	10 (71.4)	
	Distal	100 (30.9)	42 (30.7)	20 (37.7)		14 (26.4)	17 (53.1)	2 (14.3)	
	Rectosigmoid	40 (12.4)	12 (8.8)	4 (7.6)		8 (15.1)	2 (6.3)	1 (7.1)	
	Rectum	77 (23.8)	28 (20.4)	6 (11.3)	0.230	16 (30.2)	7 (21.9)	1 (7.1)	0.004
Molecular	MSS	258 (85.2)	113 (86.9)	51 (100)	0.013	40 (81.6)	30 (93.8)	12 (80.0)	0.259
characteristics,									
n (%)									
	CIMP+	72 (26.5)	34 (28.3)	16 (34.8)	0.504	14 (30.4)	8 (27.6)	8 (57.1)	0.126
KRAS mutations,	KRAS mutated	115 (35.3)	44 (32.1)	23 (42.6)	0.393	20 (37.7)	11 (33.3)	7 (46.7)	0.676
n (%)									

The survival impact of the KRAS-LCS6 variant combined with KRAS mutation status is independent of other prognostic factors

In multivariate analyses, no statistically significant differences in cause-specific survival were found for early-stage (HR 0.46; 95% CI: 0.18–1.14), stage III (HR 0.98, 95% CI: 0.55–1.74) or stage IV cases (HR 0.42; 95% CI: 0.17–1.06) with the G-allele variant as compared with wild types, although early-stage and stage IV G-allele carriers seemed to have a slightly better survival (Table 3).

Early-stage G-allele carriers with KRAS mutations seemed to have a good prognosis; none of these patients died due to CRC. In contrast, no statistically significant differences in survival were found between KRAS nonmutated early-stage (HR 0.77; 95% CI: 0.30–1.97), stage III (HR 0.95; 95% CI: 0.44–2.05) or stage IV cases (HR 0.35; 95% CI: 0.11–1.13) with the KRAS-LCS6 variant. However, stage III G-allele carriers with KRAS mutations seemed to have a poor prognosis (HR 1.52; 95% CI: 0.66–3.54) although not statistically significant.

As Dutch guidelines did not advise adjuvant treatment at the time patients were diagnosed with CRC in the NLCS, the proportion of patients that received adjuvant

treatment was very low. Within the early-stage cases, only 9% received adjuvant chemotherapy, for the advanced stage patients this was 31% for stage III and 19% for stage IV. Exclusion of treated patients did not alter our conclusions (data not shown) although it even enhanced the difference between early-stage and stage III G-allele carriers with KRAS mutations (early stage: no CRC-related deaths; stage III: HR 2.36 95% CI: 0.99–5.67) implying that stage III G-allele carriers might indeed have a worse natural course of the disease. However, this analysis is based on small patient numbers.

The survival impact of the KRAS-LCS6 variant is independent of MSI

As MSI is currently the only established molecular prognostic marker in CRC, we studied the effect of KRAS-LCS6 genotype stratified for MSI. Exclusion of patients that had an MSI tumor, which is associated with a good prognosis, did not alter our conclusions; both MSI and MSS cases with the KRAS-LCS6 G-variant had a good prognosis. In contrast, patients with the KRAS-LCS6 wild type had a poor prognosis, even if they had an MSI tumor (log-rank test, $P = 0.036$) (Fig. 3). Additional analyses stratified for sex, tumor

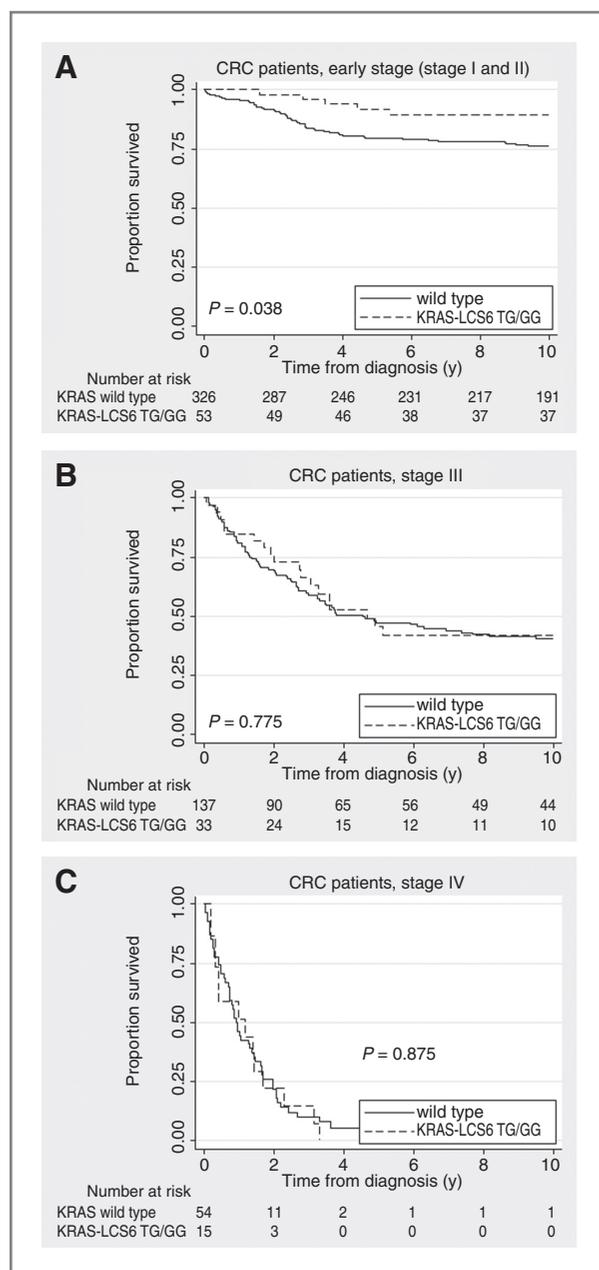


Figure 1. A, Kaplan–Meier curve for the *KRAS-LCS6* variant and cause-specific survival in early-stage (stage I and II) CRC. B, Kaplan–Meier curve for the *KRAS-LCS6* variant and cause-specific survival in stage III CRC. C, Kaplan–Meier curve for the *KRAS-LCS6* variant and cause-specific survival in stage IV CRC.

sublocation or differentiation grade within MSI patients were not possible due to limited patient numbers.

The risk of advanced stage CRC is not associated with the *KRAS-LCS6* variant

To study the possibility that the *KRAS-LCS6* G-allele predisposes for advanced stage CRC, we studied the association between *KRAS-LCS6* genotype and CRC risk. The *KRAS-LCS6*

G-allele was found in 18% of the subcohort members. For CRC, we observed a decreased risk of developing early-stage (stage I or II) CRC when carrying the *KRAS-LCS6* G-allele (RR 0.68, 95% CI: 0.49–0.94). The risk of developing advanced stage CRC (stage III or IV) was however not influenced by the *KRAS-LCS6* genotype (RR stage III: 1.02, 95% CI: 0.68–1.53; RR stage IV: 1.15, 95% CI: 0.63–2.09).

Discussion

In this study, we tested the hypothesis that a T>G variant in the *LCS6* in the 3'UTR region of *KRAS* affects prognosis in early-stage (stage I and II) CRC. The *KRAS-LCS6* G-variant was present in 16.4% of the cases whereas it is only found in 6% of world populations (13), and 12% to 15% in persons from European descent (14). We found an increased frequency of the *KRAS-LCS6* G-allele in advanced cases (early-stage 14%, 19.2%, and 21.4% in stage III and IV patients, respectively), which is comparable with previously reported frequencies in stage III (19). The G-allele was found in 18% of the subcohort members. We found a statistically significant association between the *KRAS*-variant and an increased presentation with advanced colon cancer, perhaps giving some insight into the natural biology of colon cancer in *KRAS-LCS6* variant carriers. Furthermore, we found a statistically significant increase in survival for early-stage CRC cases with the *KRAS-LCS6* G-variant; among *KRAS*-mutated patients none of the early-stage patients carrying the G-allele died from CRC. This effect was independent of other prognostic factors such as tumor differentiation or sublocation. As T4 tumors were rare in our group of early-stage cases, a higher frequency of stage IIb cases among *KRAS-LCS6* wild types is not the cause of the observed worse outcome. No statistically significant effect was seen in stage III or IV, although results suggested a slightly worse prognosis for stage III cases with the G-variant and *KRAS* mutations. In addition, we studied the effect of the *KRAS-LCS6* G-allele on CRC risk and observed a slightly decreased risk of early-stage CRC, but no effect on the risk of advanced stage CRC, suggesting that the G-allele is not associated with a higher likelihood of advanced stage CRC.

In a number of previous studies, mutations in *KRAS* have been associated with a poorer prognosis. However, we and others have recently described that results on this topic are inconsistent and the clinical relevance of these findings are unclear (4). Acquired *KRAS* mutations are however not the same as the *KRAS-LCS6* variant, which is congenital and could therefore have a different effect on tumor development, biology, and thus prognosis.

The unexpected finding that the *KRAS-LCS6* variant is associated with an increased survival in early-stage CRC is intriguing. Previous research has suggested that cellular senescence can be triggered by overexpression of oncogenic Ras and might contribute to growth cessation in premalignant or benign neoplasms (29). Tumor cell senescence has been reported in human cancers, and premalignant colon adenomas display features of senescence as well (30). Previous studies have often suggested that

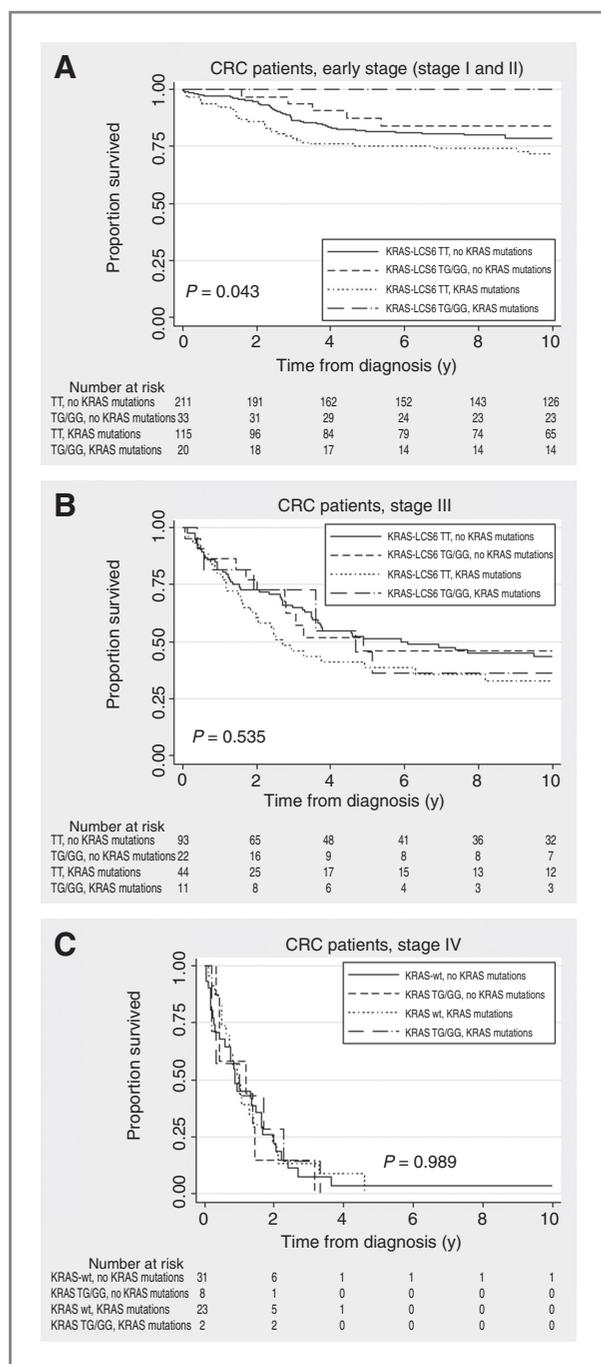


Figure 2. A, Kaplan–Meier curve for the KRAS-LCS6 variant, KRAS mutations and cause-specific survival in early-stage (stage I and II) CRC, $P = 0.875$. B, Kaplan–Meier curve for the KRAS-LCS6 variant, KRAS mutations and cause-specific survival in stage III CRC. C, Kaplan–Meier curve for the KRAS-LCS6 variant, KRAS mutations and cause-specific survival in stage IV CRC.

oncogene-induced senescence plays a role in premalignant lesions only. Nevertheless, recent evidence suggests that physiologic levels of *KRAS* can induce senescence in the absence of the transcription factor Wilms tumor 1 (*WT1*) and lung cancer patients with a high *KRAS* gene

expression had a good prognosis if they had decreased expression of *WT1* related genes (31). These results imply that other molecular factors can be involved in the determination of cell fate, and that oncogene-induced senescence can occur after an altered expression of other genetic or epigenetic targets. Hypothetically, this could also play a role in CRC, and the *KRAS-LCS6* genotype could either lead to an advanced stage tumor, or an early-stage tumor with a better prognosis based on the other (epi)genetic markers that are affected. However, evidence on this concept is scarce and more research is needed to elucidate whether our findings can be explained by oncogene-induced senescence.

Similar to *KRAS* mutations, we observed a better outcome for early-stage (stage I and II) cases with the *KRAS-LCS6* G-variant and *BRAF* mutations or *RASSF1A* hypermethylation, 2 other genes involved in the Ras signaling pathway. *BRAF*-associated senescence has previously been reported to occur in melanoma (32) but a possible role of *RASSF1A* in oncogene-induced senescence is currently unknown. As in our population both events are less common, statistical significance was not reached. When combining these (epi)genetic events, the better outcome of patients with the variant G-allele and *KRAS*, *BRAF*, or *RASSF1A* alterations was even more enhanced. Along this line, we hypothesize that Ras overexpression due to the *KRAS-LCS6* G-allele, in combination with (epi)genetic alterations in genes from the Ras pathway, could induce senescence in early-stage CRC thereby influencing survival. For advanced cases on the other hand, an increasing number of molecular pathways are affected that all have a role in prognosis. Although intriguing, this is only speculative; more research is needed to determine whether the *KRAS-LCS6* G-variant and (epi)genetic alterations in Ras-associated genes can lead to oncogene-induced senescence in early-stage CRC.

Several studies have previously shown a tumor growth suppression effect of the *let-7* miRNA (10–12, 33–35), and lower *let-7* expression and higher *KRAS* levels in the presence of the *KRAS-LCS6* G-variant (13). Following this, it would be expected that patients with the *KRAS-LCS6* variant have a worse prognosis and this has been shown for oral cancer (17). For CRC, there are 2 published reports studying the effect of *KRAS-LCS6* genotype (19, 20). One reports a poor survival among a small population of irinotecan-refractory metastatic patients with the *KRAS-LCS6* G-variant, and an association with *KRAS* mutations and the absence of *BRAF* mutations (19), these findings could not be replicated in our study. The second reports a better response to cetuximab in metastatic CRC and a longer survival in patients with the G-variant without *KRAS* mutations, but not statistically significant (20). We also observe a slightly better prognosis in stage IV G-allele carriers, although not statistically significant, but our group of stage IV patients is small rendering instable results. Although previous studies used germline tissue to assess the *KRAS-LCS6* genotype, we used tumor DNA to assess genotype. However, it has previously been well documented that genotype of

Table 3. HRs and 95% CI for cause-specific mortality and clinicopathologic and the KRAS-LCS6 variant in 734 CRC cases from the Netherlands Cohort Study on diet and cancer

	Early stage (stage I and II) CRC	Stage III CRC	Stage IV CRC
KRAS-LCS6 variant	0.46 (0.18–1.14)	0.98 (0.55–1.74)	0.42 (0.17–1.06)
KRAS-LCS6 variant without KRAS mutations	0.77 (0.30–1.97)	0.95 (0.44–2.05)	0.35 (0.11–1.13)
KRAS-LCS6 variant with KRAS mutations	No CRC-related deaths	1.52 (0.66–3.54)	0.60 (0.19–1.91)
Sex (male)	0.97 (0.60–1.57)	0.92 (0.59–1.45)	0.85 (0.44–1.64)
Age at diagnosis	0.99 (0.94–1.05)	1.01 (0.96–1.06)	1.02 (0.93–1.10)
Grade	1 (reference)	1 (reference)	1 (reference)
	2	0.91 (0.34–2.45)	2.14 (0.28–16.38)
	3	0.77 (0.09–6.72)	1.90 (0.52–6.94)
	4	–	4.17 (0.72–24.05)
Sublocation of the tumor	Proximal	1 (reference)	1 (reference)
	Distal	0.76 (0.41–1.43)	0.67 (0.37–1.19)
	Rectosigmoid	0.32 (0.11–0.94)	0.60 (0.24–1.48)
	Rectum	0.49 (0.18–1.36)	0.24 (0.08–0.69)

normal and tumor tissue is the same for the *KRAS-LCS6* variant (13).

The conflicting results in early and advanced stage CRC also raises questions on the origin and progression of tumors in different cancer stages, and whether early-stage CRC might develop through a molecular distinct pathway as compared with advanced stage. Our results indicate that the *KRAS-LCS6* variant is more common among cases with advanced stage disease, however, those patient that are diagnosed early with the *KRAS-LCS6* variant seem to have a more advantageous outcome. This might imply a different biology in early-stage as compared with advanced stage cases. In addition, our finding that early-stage *KRAS-LCS6* wild-type patients have a poor prognosis, even if they have a

MSI tumor, might indicate that these patients are possibly in need of (additional) adjuvant treatment. However, further research including randomized clinical trials, is needed to assess whether these early-stage patients with a poor prognosis would benefit from additional adjuvant treatment. Up until now, MSI has been considered to be a marker for good prognosis (36) however, our data suggest a better outcome for *KRAS-LCS6* G-allele carriers independent of MSI status. Even though our study is the largest study on the *KRAS-LCS6* genotype in CRC up until now, patient numbers in specific subgroups are still small. Larger, prospective studies and randomized clinical trials are needed to validate the potential role of *KRAS-LCS6* genotype as a prognostic biomarker in therapy decision-making.

In conclusion, our assessment of the influence of the *KRAS-LCS6* G-variant in early-stage CRC cases showed a better outcome for early-stage G-allele carriers with *KRAS* mutations. Although the population used in this study is among the largest studies that have been done on the *KRAS-LCS6* variant, subgroups were small. However, this population is the only group studied to date that is generally untreated, and for the first time gives insight into the natural biology of CRC with the LCCS6 variant. Future studies validating our results are however needed. Nevertheless, our data should be regarded as hypothesis generating providing a first indication that the *KRAS-LCS6* genotype is a possible prognostic biomarker for early-stage CRC that can be used to identify CRC patients with a good prognosis.

Disclosure of Potential Conflicts of Interest

J.B. Weidhaas has a consultant and advisory role in MiraDX, her husband is a board member of MiraDX (uncompensated), and she has stock ownership of MiraDX. The other authors disclosed no potential conflicts of interest.

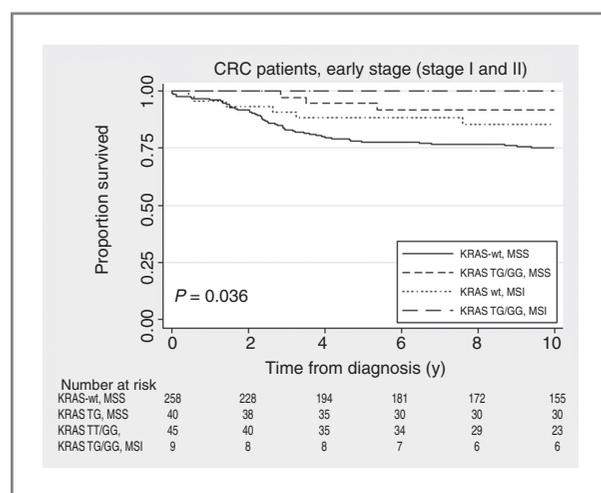


Figure 3. Kaplan-Meier curve for the *KRAS-LCS6* variant, MSI status and cause-specific survival in early-stage (stage I and II) CRC.

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