Regulation of KRAS protein expression by *miR-544* and *KRAS-LCS6* polymorphism in wild-type *KRAS* sporadic colon adenocarcinoma

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ABSTRACT

Colorectal carcinoma (CRC) results from the accumulation of genetic mutations and alterations in signaling pathways. *KRAS* is mutated in 40% of CRC cases and is involved in increased tumor cells proliferation and survival. Although *KRAS* mutations are a dominant event in CRC tumorigenesis, increased wild-type KRAS expression has a similar effect on accelerated tumor growth. In this study, we investigated the KRAS status in correlation with clinicopathological features in sporadic CRC and more importantly the role of *let-7a-5p* and *miR-544a-3p* in the regulation of wild-type KRAS protein expression in the tumor center (T1) and invasive tumor front (T2).

Analysis showed that 39.1% of tumor samples had *KRAS* mutations. In wild-type *KRAS* tumors, 62.0% were positive for KRAS protein expression and there was a higher percentage of KRAS positive tumor cells and a higher intensity of immunohistochemical reaction in T2 than in T1 samples. This could not be attributed to differences in *KRAS* mRNA levels, suggesting regulation via *miR-544a-3p* expression which was significantly decreased in T2 samples. Furthermore, we demonstrated that tumor samples carrying the *KRAS-LCS6* variant allele had significantly higher protein expression of the wild-type KRAS.

Our results suggest the role of the *KRAS-LCS6* polymorphism and *miR-544a-3p* expression in the regulation of wild-type KRAS protein expression in sporadic CRC.

Keywords: colon adenocarcinoma, immunohistochemistry, KRAS, *let-7a*, *miR-544a*

INTRODUCTION

Colorectal carcinoma (CRC) is the third most commonly diagnosed cancer worldwide [1], and although advances have been made in the development and use of antitumor therapies [2], the mortality rate remains high [3]. KRAS is one of the early and key players in CRC tumorigenesis involved in the increased proliferation of tumor cells. Although *KRAS* mutations, most commonly located in codon 12 or 13 [4], are considered the dominant event in CRC tumorigenesis, it has been shown that overexpression of the wild-type (WT) KRAS in both cancer cell lines and patient tumors can also induce tumor initiation, progression, and maintenance [5-7]. However, a comprehensive review of the literature reveals that few studies have addressed KRAS protein regulation, particularly in relation to wild-type *KRAS* CRC.

Increasing interest in non-coding RNAs revealed that KRAS protein expression and their activity can be modulated by short regulatory microRNA (miRNA) molecules [8]. As with most protein-coding genes, the *KRAS* sequence contains binding sites for several different miRNAs that can have a strong regulatory effect on KRAS expression [8-10]. One of the first miRNAs shown to affect *KRAS* expression *in vivo* was *let-7a* [11]. In CRC, *let-7a* is one of the most frequently downregulated miRNAs. *In vitro* data showed that decreased *let-7a* expression induced higher KRAS expression and increased growth and proliferation in human colon cancer cells [8]. In addition to *let-7a*, there are over 300 other miRNAs with predicted binding sites in the *KRAS* 3'-UTR whose functional roles have not been thoroughly investigated. Although there is no clear consensus on the criteria that should be used to determine miRNAs that bind in the 3`UTR of target gene, we used three bioinformatics algorithms TargetScan (http://www.targetscan.org) Ensembl (http://www.ensembl.org) and miRBase (http:// http://www.mirbase.org/). From the results obtained, we decided to investigate *microRNA-544a-3p* (*miR-544a-3p*), whose role in tumorigenesis of gliomas [12], and gastric [13] and ovarian cancer [14] has been demonstrated by previously published data.

Single-nucleotide polymorphisms (SNP) in miRNA recognition sites in the 3′-UTRs of mRNA have recently attracted attention because they can affect the binding efficiency of their target mRNA and thus regulate protein expression [15-17]. A functional SNP *KRAS-LCS6* (rs61764370 T/G) in the *let-7a-5p* binding site within the *KRAS* 3'-UTR has been described and associated with better prognosis and response to anti-EGFR-based therapy in CRC [16, 18-20]. Using the PolymiRTS database (http://compbio.uthsc.edu/miRSNP), we identified another less studied SNP *KRAS* rs10771184 A/T in the *miR-544a-3p* binding site that was associated

with increasedsurvival in ovarian cancer [14]. However, the data regarding the role of miRNAs *let-7a* and *miR-544a-3p* and their binding sites in CRC is still controversial due to lack of reproducibility of the findings [21-23].

The aim of the current study was to investigate the correlation between *KRAS* mutation status and clinicopathological features, focusing on sporadic wild-type KRAS colon tumors stratified by KRAS protein expression. In addition, we aimed to analyze *let-7a-5p and miR-544a-3p* expression as well as rs61764370 (T/G) and rs10771184 (A/T) variants and their role on KRAS protein expression in wild-type KRAS CRC. KRAS signaling pathway plays a role in the tumor cells invasiveness of and there is increasing evidence that epithelial-to-mesenchymal transition can be regulated by miRNAs [24-26]. Therefore, we decided to investigate the role of *miR-544a-3p* and *let-7a-5p* in the regulation of KRAS protein expression in the tumor center (T1) and invasive tumor front (T2) to improve our understanding of miRNA biology in CRC.

MATERIALS AND METHODS

Patients and Samples

This study included 110 patients with sporadic colon adenocarcinoma with negative family history for hereditary cancer. Tumor and adjacent normal colon tissue were obtained from the Clinical Hospital Merkur. The study included 66 male and 44 female patients with a mean age of 64.8 years (age range 35 and 91 years).

All specimens, tumor- and adjacent histologically normal colonic mucosal tissue (15 cm from the tumor), were obtained by routine clinical procedures, and the diagnosis was confirmed histopathologically. None of the patients underwent preoperative radiation or chemotherapy. The freshly resected specimens were frozen in liquid nitrogen and stored in Human Tumor Bank[27] at -80°C for DNA and RNA extraction. Additional tumor and normal mucosa samples from patients included in the study were fixed in formalin and immersed in paraffin. Sections were 3μm thick and mounted on silanized glass slides for immunohistochemistry. In this study, two samples were obtained from each tumor, one tumor center (T1) and one invasive tumor front (T2). Prior to inclusion in the study, each slide was verified by a pathologist (A.Š.). All slides were examined with routine hematoxylin and eosin staining. Information on patients` age and sex, tumor size, differentiation, Dukes' stage, tumor budding, and location was collected.

The study was approved by the Ethics Committee of Clinical Hospital Merkur, Zagreb (on May 24, 2016, UR. BR. 03/1-4723), and was conducted according to the ethical standards of Helsinki Declaration. Written informed consent was obtained from all patients included in the study.

Detection of KRAS Mutations

DNA was extracted from two fresh frozen samples from each colon adenocarcinoma, tumor center (T1) and invasive tumor front sample (T2). DNA extraction of frozen tissue was performed by proteinase K digestion and phenol-chloroform extraction. *KRAS* mutation status of the tumors was determined by genotyping codons 12 and 13 using the PCR-RFLP method, as previously described [28].

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue sections from sporadic colon adenocarcinomas were stained for KRAS protein with a rabbit polyclonal antibody (HPA049830) (Sigma-Aldrich, Germany). Negative controls were performed by omitting the primary antibody. After deparaffinization in xylene, slides were rehydrated in ethanol and washed in phosphate-buffered saline. Antigen retrieval was performed by microwave heating in citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0). Endogenous peroxidase activity was quenched by incubation in methanol containing 3% hydrogen peroxide (Sigma-Aldrich, Germany) for 15 min. Nonspecific binding was blocked by applying DAKO Protein Block Serum-Free (DAKO, Denmark) in a humidified chamber for 10 min at room temperature. Slides were blotted, and primary mouse monoclonal antibody at a concentration of 5 μg/ml was applied for 1 h at room temperature. The slides were then washed three times in phosphate-buffered saline. The DAKO EnVision^{TM} + System, HRP (DAB) (DAKO, Denmark) was used to visualize positive reactions according to the manufacturer's instructions. Slides were counterstained with hematoxylin, dehydrated, and mounted in Entellan (Sigma-Aldrich, Germany). Each slide was independently scored by two observers (A.Š. and S.K.) as positive (if more than 10% of the cells showed positive staining) or negative for KRAS in the entire tumor area.

The relative intensity of cell immunostaining assessed semiquantitatively to indicate no staining (0), weak staining (1), moderate staining (2), or strong staining (3).

SNP Genotyping

Two SNPs in miRNA-binding sites within the 3'UTR of the *KRAS* gene, which were predicted to affect the binding of *let-7a-5p* and *miR-544a-3p* were analyzed. *KRAS-LCS6* T/G (rs61764370) genotyping was performed by PCR-RFLP analysis[29]. For PCR amplification, forward primer 5'-TTA GGA GAG ACG GGG TTT CA-3' and reverse primer 5'-AAA TGA GTT CTG CAA AAC AGG-3' were used. For restriction fragment length polymorphism analysis, 5μl of PCR products were digested overnight with restriction enzyme TfiI (New England Biolabs) at 37ºC. Allelic discrimination was performed by 10% non-denaturing polyacrylamide gel electrophoresis. Gels were stained with silver.

The SNP in the binding site for *miR-544a-3p, KRAS* rs10771184 A/T was analyzed using Custom Taqman® SNP Genotyping Assay (Applied Biosystems) according to the manufacturer's protocol.

For quality control, 15% of randomly selected samples were analyzed a second time, and no discrepancies were detected. Control samples covering three possible SNP genotypes and no template control were run in parallel with the tested samples in each experiment.

mRNA and miRNA Expression Analysis

Snap-frozen fresh samples of colon adenocarcinoma and adjacent normal tissue samples were used for total RNA extraction with Trizol according to the manufacturer's protocol. RNA samples were subjected to quantification and quality control and 50 samples with the highest quality were selected for mRNA and miRNA analysis. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for both mRNA and miRNA expression detection according to the manufacturer's protocols. Random hexamers were used for mRNA conversion and analysis, whereas specific primers were used for *let-7a-5p*, *miR-544a-3p*, and *RNU48* analyzes (000377, 002265, and 001006, respectively; Applied Biosystems) according to the manufacturer's protocol.

Predeveloped Taqman® Gene Expression Assay was used for *KRAS* mRNA expression quantification (Hs00364284_g1, Applied Biosystems), and *ACTB* (Hs01060665_g1, Applied Biosystems) as an endogenous control. Taqman® MicroRNA assays were used for *let-7a-5p* and *miR-544a-3p* (000377 and 002265, respectively; Applied Biosystems) quantification and *RNU48* (001006, Applied Biosystems) as an endogenous control. The results of mRNA and miRNA expression were calculated using the comparative Ct method[30].

Statistical Analysis

Statistical analyzes were performed using the GraphPad Prism statistical package (GraphPad Software). For continuous data, normality test was performed before analysis[31]. One-way analysis of variance (ANOVA) with Bonferroni correction was used to compare the percentage of immunohistochemically positive KRAS cells and mRNA and miRNA expression between N, T1 and T2 groups. For *KRAS* mutation status or IHC analysis and further correlation with clinicopathological features, a contingency table with Fisher’s exact test was used to calculate statistical significance. Student`s t-test was used to compare mRNA and miRNA expression between T1 and T2 groups. Data are presented as mean ± s.e.m or box-and-whisker plots (5-95 percentiles). Values of $*p<0.05$, $**p<0.01$, and $***p<0.001$ were considered statistically significant.

RESULTS

KRAS Mutations

One hundred and ten samples of sporadic colon adenocarcinomas were analyzed for mutations in codons 12 and 13. Two samples from each tumor were analyzed, the tumor center (T1) and the invasive tumor front (T2). Analysis showed that 43 (39.1%) tumors were positive for *KRAS* mutations. *KRAS* codon 12 mutation was detected in 39 (90.7%) and *KRAS* codon 13 mutation in 4 (9.3%) tumors. Each tumor was either negative or positive for *KRAS* mutation in both tumor samples (T1 and T2) analyzed. The results of *KRAS* mutation detection were correlated with the clinicopathological characteristics of the patients and tumors. There was no statistically significant difference between wild-type and mutation-positive *KRAS* tumors by clinicopathological characteristics, except for from tumor budding (p=0.038) (Table 1).

KRAS Protein Expression

Sixty-five samples of sporadic colon adenocarcinomas (50 samples negative, and 15 samples positive for *KRAS* mutations) were analyzed by immunohistochemistry for the expression of KRAS protein. From each patient, we analyzed the adjacent normal tissue (N), tumor center (T1), and invasive tumor front (T2). The staining pattern observed was identical in all tumors and was visualized by membranous and/or cytoplasmic staining of tumor cells (Figure 1). All tumors with more than 10% positive tumor cells were classified as KRAS positive.

For wild-type *KRAS* tumors, 19 (38.0%) samples were negative and 31 (62.0%) were positive for KRAS protein expression. KRAS-protein expression was higher in both percentage of positive tumor cells (p=0,04) (Figure 2A) and intensity (p=0,03) (Table 2) in T2 samples compared to N. There was also a statistically significant difference between T1 and T2 in the percentage of positive tumor cells (p=0,04) (Figure 2A) and in the intensity of the immunohistochemical reaction to KRAS protein (p=0,03; Table 2). In addition, negative, or weak KRAS protein expression was also found in adjacent normal tissue (Figure 2A, Table 2).

KRAS protein expression was detected in all tumors that were positive for *KRAS* mutations. These tumors had an overall higher percentage of KRAS positive cells and immunohistochemical intensity than tumors negative for *KRAS* mutations. There was no statistically significant difference between N, T1 and T2 samples in the percentage of positive tumor cells (Figure 2B). The KRAS protein expression was higher in T2 compared to N in

immunohistochemical intensity (p=0.003; Table 2). Moderate KRAS protein expression was also found in adjacent normal tissue (Figure 2B, Table 2).

The results of KRAS protein expression analysis in wild-type *KRAS* tumors correlated with the clinicopathological characteristics of the patients and tumors showed no statistical significance (Table 3).

KRAS mRNA, let-7a-5p, and mir-544a-3p miRNA Expression

Because possible base pairing between *KRAS mRNA and miR-544a-3p and let-7a-5p* was predicted using IntaRNA 2.0 software (Figure 3), we examined *KRAS* mRNA, *miR-544a-3p,* and *let-7a-5p* expression in fifty samples of wild-type *KRAS* colon adenocarcinoma (T1 and T2) and adjacent normal tissue. The analysis showed no statistically significant difference in *KRAS* mRNA or *let-7a-5p* miRNA expression between adjacent normal and tumor tissues (T1 and T2) (p=0.98; p=0.52, respectively) (Figure 4A and 4B). However, the expression of *miR-544a-3p* was significantly decreased in the invasive tumor front compared to that in the corresponding adjacent normal tissue (p=0.02, Figure 4C).

Correlation of mRNA and miRNAs Expression with KRAS Protein Expression

We also examined the correlation between KRAS protein expression and *KRAS* mRNA, *let-7a-5p* and *miR-544a-3p* expression in wild-type *KRAS* tumor tissues. There was no difference between *KRAS* mRNA or *let-7a-5p* expression and KRAS protein expression (p=0.32; p=0.92, respectively; Figure 5A and 5B). Nevertheless, *miR-544a-3p* expression was significantly lower in T2 than in T1 only in tumors that expressed KRAS protein by immunohistochemistry (p=0.04) (Figure 5C).

SNPs in miRNA Binding Sites

All wild-type *KRAS* samples were genotyped for two SNP sites within 3'UTR of the *KRAS* gene, KRAS-LCS6 T/G (rs61764370) polymorphism in the *let-7a-5p* binding site and *KRAS* rs10771184 A/T polymorphism in the *miR-544a-3p* binding site.

The genotype distribution of *KRAS*-LCS6 T/G polymorphism in wild-type *KRAS* CRC was as follows: wild-type homozygous genotype TT was detected in 44 (88%), heterozygous genotype TG in 5 (10%), and variant homozygous genotype GG in 1 (2.0%) sample. The distribution of *KRAS* rs10771184 A/T polymorphism genotypes in wild-type *KRAS* was as follows: wild-type homozygous genotype AA was detected in 12 (24%), heterozygous genotype AT in 26 (52%), and variant homozygous genotype TT in 12 (24.0%) samples.

The genotype frequencies of both SNPs analyzed in 50 sporadic wild-type *KRAS* colon adenocarcinomas and stratified by KRAS protein status are shown in Table 4. The results showed no statistically significant association between *KRAS* rs10771184 A/T polymorphism and KRAS protein expression. However, a statistically significant association between KRAS protein expression and *KRAS-LCS6* T/G polymorphism was found. All tumor samples of variant allele carriers (TG and GG) were positive to KRAS protein and variant allele G was only present in KRAS protein-positive tumors ($p=0.04$, $p=0.03$ respectively).

DISCUSSION

Colorectal adenocarcinoma is a result of molecular genetic alterations in many oncogenes and tumor suppressor genes[32]. Activating KRAS mutations that drive cell proliferation, migration and invasion occur in approximately 50% of colorectal carcinomas [33, 34]. Although the mutation in KRAS is an early and dominant event, amplification and overexpression of KRAS, leading to activation of the KRAS/MAPK pathway, can also be observed in colorectal cancer patients [35]. Considering that KRAS mutations have been thoroughly investigated in CRC [36-38], we focused our study on patients negative for the most common *KRAS* activating mutations and showed that wild-type KRAS protein expression could be regulated via *miR-544a-3p* expression and *let-7a-5p KRAS-LCS6* T/G polymorphism.

We analyzed associations between *KRAS* mutation status and clinicopathological features in 110 sporadic colorectal tumors. The incidence of codon 12 and codon 13 mutations in *KRAS* was 40% and is comparable to the previously published study by Liu et al.[39], in which approximately 40% of CRC had KRAS exon 2 mutation[40]. In our study, we found that tumors with *KRAS* mutations correlated with higher tumor budding, an independent prognostic marker for lymph node metastasis and survival for CRC [41-43], a phenomenon also observed by other groups [44, 45]. However, unlike others [46, 47], we found no correlation with other clinicopathological characteristics such as age, sex, tumor size or left/right side location, histological grade, or Dukes` stage. This discrepancy could be partially explained by the fact that we did not address *KRAS* mutation subtypes due to the limited number of patients with codon 13 mutation. Patients with BRAF mutation-positive tumors were also excluded from our study to obtain a more accurate correlation between clinicopathological features and wild-type CRC.

KRAS mutation status is an important pharmacogenetic marker for response to anti-epidermal growth factor receptor (EGFR) antibody therapy in CRC patients[48]. However, little attention has been paid to the function and regulation of wild-type *KRAS* in the context of CRC. Therefore, we performed an immunohistochemical analysis of KRAS protein levels in *KRAS*-mutated and wild-type tumors, assuming that *KRAS*-mutated tumors would have overexpression of KRAS protein. Indeed, in samples in which *KRAS* was mutated, there was a higher percentage of KRAS positive cells and a higher staining intensity, which is in agreement with the results of other authors who described KRAS protein overexpression in CRCs positive for *KRAS* mutations [49, 50]. Regarding the protein expression of the wild-type KRAS, we detected a high KRAS expression in some samples, while it was completely absent in others.

Similar results were also observed in another study trying to see if immunohistochemistry correlated with mutation status[50]. The relationship between KRAS positive or negative immunohistochemical expression and tclinicopathological features in wild-type *KRAS* tumors showed no significant correlation. And because only few studies have addressed wild-type KRAS immunohistochemical protein expression, especially in colorectal carcinoma, our results are hardly comparable with others. However, it is important to emphasize that we found for the first time that the invasive tumor front had higher KRAS protein expression than the corresponding tumor center in wild-type *KRAS* tumor samples. Nevertheless, this could not be attributed to differences in *KRAS* mRNA levels, suggesting other posttranscriptional mechanisms.

Since the discovery of miRNA, its involvement in gene regulation of various pathological processes, such as cancer development, has been recognized [51, 52]. The existence and oncogenic or tumor suppressive effects of these non–protein coding RNAs have also been recognized in colorectal carcinoma [53, 54]. In our study, we focused on one of the first miRNAs discovered, that was shown to negatively regulate KRAS in CRC, *let-7a-5p*. Although *let-7a-5p* has been shown to be reduced in CRC, we did not obtain these results either in the tumor center or at the invasive tumor front, suggesting that it does not play a role in wild-type KRAS protein expression. In our study, we also included the much less studied potential *KRAS*-regulating miRNA, *miR-544a-3p*. Its role in cancer has been established, but not in the context of KRAS. Here, we show that decreased levels of *miR-544a-3p* at the invasive tumor front, in comparison to tumor center and adjacent normal colon tissue, correlate with higher KRAS protein expression compared to the tumor center and adjacent normal intestinal tissue. Moreover, we demonstrate significantly lower *miR-544a-3p* expression specifically in wild-type *KRAS* T2 tumor specimens showing positive immunohistochemical KRAS protein expression. Since the reduced expression of *miR-544a-3p* correlates with higher KRAS expression, we hypothesize that it may have a role in wild-type KRAS protein regulation and that these changes likely represent a mechanism for strengthening the already activated RAS signaling.

Genomic profiling allowed us to identify and increase the importance of SNPs in miRNA activity. The whole concept of miRNA-mRNA interaction and regulation of target protein expression is based on specific sequence complementarity. Therefore, it is susceptible to disruption by sequence changes such as SNPs[55]. In the last decade, many studies have been published analyzing the prognostic and predictive roles of *KRAS* SNPs rs10771184 and

rs61764370 in miRNA binding sites of the *KRAS* gene. And while several studies emphasized the potential role of these SNPs as molecular biomarkers in various tumors for disease susceptibility[56, 57], prognosis[18, 58], survival[59, 60], and prediction of drug response [61], others found no correlation[62, 63]. However, to the best of our knowledge, we are the first study to publish an association between these two polymorphisms and KRAS protein expression. We found that tumor samples carrying the *KRAS-LCS6* variant allele express KRAS protein, suggesting that the variant allele G may alter the binding of *let-7a-5p* to the *KRAS* mRNA, resulting in the increase of KRAS protein. This is consistent with previously published results that there is higher *KRAS* mRNA expression in colorectal cancer cells transfected with the full-length *KRAS* 3′UTR variant G allele[29]. In the same way we also investigated the role of *KRAS* SNP rs10771184 A>T in the *miR-544a-3p* binding site, but we found no association between KRAS protein expression and the presence of a variant T allele.

In this study, we recognized the importance of *KRAS-LCS6* polymorphism in *let-7a-5p* binding site and *miR-544a-3p* on the regulation of KRAS protein; however, there are still some limitations of our study. First, the relatively small number of patients and second, the lack of *in vitro* cell-based analysis of the effect of miRNAs and polymorphisms in their binding sites on KRAS protein expression. Therefore, future studies are needed to confirm our results and to identify possible mechanisms of action of *let-7a-5p* and *miR-544a-3p* on KRAS protein expression in CRC. In addition, the expression levels of miRNAs should be examined on a larger number of samples to draw more reliable conclusions.

CONCLUSIONS

In conclusion, our results suggest the role of *KRAS-LCS6* polymorphism and *miR-544a-3p* in tumorigenesis from sporadic CRC negative to KRAS mutation. Our study showed that tumor samples carrying the *KRAS-LCS6* variant allele have significantly higher protein expression of KRAS suggesting that the SNP in the *let-7a-5p* binding region of the KRAS gene is associated with higher KRAS protein levels in wild-type *KRAS* tumors. Furthermore, our results suggest that *miR-544a-3p* has a tumor-suppressive effect and is a potential regulator of wild-type KRAS protein expression in the invasive tumor front which may be associated with the progression of this malignant tumor. It is expected that further studies will provide new insights into *miR-544a-3p* as a potential prognostic marker in colon adenocarcinoma.

DECLARATIONS

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Ethics approval and informed consent to participate

Written informed consent was obtained from all patients included in the study. The study was approved by the ethics committee of Merkur Clinical Hospital, Zagreb and Medical School, University of Zagreb, and was performed under the ethical standards of the Helsinki Declaration.

Authors' contributions

S. Marinović: Investigation, Formal analysis, Writing - Original Draft, **A. Škrtić**: Resources, Formal analysis, Visualization, **T. Catela Ivković**: Investigation, Formal analysis, **M. Poljak**: Resources, **S. Kapitanović**: Conceptualization, Funding acquisition, Writing- Reviewing and Editing.

LITERATURE

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.
2. Mondaca S, Yaeger R. Colorectal cancer genomics and designing rational trials. Ann Transl Med. 2018;6(9):159.
3. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021.
4. Tsilimigras DI, Ntanasis-Stathopoulos I, Bagante F, Moris D, Cloyd J, Spartalis E, et al. Clinical significance and prognostic relevance of KRAS, BRAF, PI3K and TP53 genetic mutation analysis for resectable and unresectable colorectal liver metastases: A systematic review of the current evidence. Surg Oncol. 2018;27(2):280-8.
5. Guerrero S, Casanova I, Farre L, Mazo A, Capella G, Mangues R. K-ras codon 12 mutation induces higher level of resistance to apoptosis and predisposition to anchorage-independent growth than codon 13 mutation or proto-oncogene overexpression. Cancer Research. 2000;60(23):6750-6.
6. Horsch M, Recktenwald CV, Schadler S, Hrabe de Angelis M, Seliger B, Beckers J. Overexpressed vs mutated Kras in murine fibroblasts: a molecular phenotyping study. British Journal of Cancer. 2009;100(4):656-62.
7. Zhou B, Der CJ, Cox AD. The role of wild type RAS isoforms in cancer. Seminars in Cell & Developmental Biology. 2016;58:60-9.
8. Roncarati R, Lupini L, Shankaraiah RC, Negrini M. The Importance of microRNAs in RAS Oncogenic Activation in Human Cancer. Front Oncol. 2019;9:988.
9. You C, Liang H, Sun W, Li J, Liu Y, Fan Q, et al. Deregulation of the miR-16-KRAS axis promotes colorectal cancer. Sci Rep. 2016;6:37459.
10. Kim M, Slack FJ. MicroRNA-mediated regulation of KRAS in cancer. J Hematol Oncol. 2014;7:84.
11. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by the let-7 microRNA family. Cell. 2005;120(5):635-47.
12. Ma R, Zhang G, Wang H, Lv H, Fang F, Kang X. Downregulation of miR-544 in tissue, but not in serum, is a novel biomarker of malignant transformation in glioma. Oncol Lett. 2012;4(6):1321-4.
13. Yanaka Y, Muramatsu T, Uetake H, Kozaki K, Inazawa J. miR-544a induces epithelial-mesenchymal transition through the activation of WNT signaling pathway in gastric cancer. Carcinogenesis. 2015;36(11):1363-71.
14. Liang D, Meyer L, Chang DW, Lin J, Pu X, Ye Y, et al. Genetic variants in MicroRNA biosynthesis pathways and binding sites modify ovarian cancer risk, survival, and treatment response. Cancer Research. 2010;70(23):9765-76.
15. Gottmann P, Ouni M, Zellner L, Jahnert M, Rittig K, Walther D, et al. Polymorphisms in miRNA binding sites involved in metabolic diseases in mice and humans. Sci Rep. 2020;10(1):7202.
16. Jacinta-Fernandes A, Xavier JM, Magno R, Lage JG, Maia AT. Allele-specific miRNA-binding analysis identifies candidate target genes for breast cancer risk. NPJ Genom Med. 2020;5:4.
17. Gholami M, Larijani B, Sharifi F, Hasani-Ranjbar S, Taslimi R, Bastami M, et al. MicroRNA-binding site polymorphisms and risk of colorectal cancer: A systematic review and meta-analysis. Cancer Med. 2019;8(17):7477-99.
18. Sclafani F, Chau I, Cunningham D, Peckitt C, Lampis A, Hahne JC, et al. Prognostic role of the LCS6 KRAS variant in locally advanced rectal cancer: results of the EXPERT-C trial. Annals of Oncology. 2015;26(9):1936-41.
19. Ryan BM, Robles AI, Harris CC. KRAS-LCS6 genotype as a prognostic marker in early-stage CRC--letter. Clinical Cancer Research. 2012;18(12):3487-8; author reply 9.

20. Usher-Smith JA, Walter FM, Emery JD, Win AK, Griffin SJ. Risk Prediction Models for Colorectal Cancer: A Systematic Review. Cancer Prev Res (Phila). 2016;9(1):13-26.
21. Dou R, Nishihara R, Cao Y, Hamada T, Mima K, Masuda A, et al. MicroRNA let-7, T Cells, and Patient Survival in Colorectal Cancer. Cancer Immunol Res. 2016;4(11):927-35.
22. Luu C, Heinrich EL, Duldulao M, Arrington AK, Fakih M, Garcia-Aguilar J, et al. TP53 and let-7a micro-RNA regulate K-Ras activity in HCT116 colorectal cancer cells. PLoS One. 2013;8(8):e70604.
23. Sha D, Lee AM, Shi Q, Alberts SR, Sargent DJ, Sinicrope FA, et al. Association study of the let-7 miRNA-complementary site variant in the 3' untranslated region of the KRAS gene in stage III colon cancer (NCCTG N0147 Clinical Trial). Clin Cancer Res. 2014;20(12):3319-27.
24. Fang C, Li Y. Prospective applications of microRNAs in oral cancer. Oncol Lett. 2019;18(4):3974-84.
25. Ghosh RD, Pattatheyil A, Roychoudhury S. Functional Landscape of Dysregulated MicroRNAs in Oral Squamous Cell Carcinoma: Clinical Implications. Front Oncol. 2020;10:619.
26. Paterson EL, Kazenwadel J, Bert AG, Khew-Goodall Y, Ruszkiewicz A, Goodall GJ. Down-regulation of the miRNA-200 family at the invasive front of colorectal cancers with degraded basement membrane indicates EMT is involved in cancer progression. Neoplasia. 2013;15(2):180-91.
27. Spaventi R, Pecur L, Pavelic K, Pavelic ZP, Spaventi S, Stambrook PJ. Human tumour bank in Croatia: a possible model for a small bank as part of the future European tumour bank network. Eur J Cancer. 1994;30A(3):419.
28. Nollau P, Moser C, Weinland G, Wagener C. Detection of K-ras mutations in stools of patients with colorectal cancer by mutant-enriched PCR. International Journal of Cancer. 1996;66(3):332-6.
29. Saridaki Z, Weidhaas JB, Lenz HJ, Laurent-Puig P, Jacobs B, De Schutter J, et al. A let-7 microRNA-binding site polymorphism in KRAS predicts improved outcome in patients with metastatic colorectal cancer treated with salvage cetuximab/panitumumab monotherapy. Clinical Cancer Research. 2014;20(17):4499-510.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
31. D'Agostino RB, Belanger A, D'Agostino RB, Jr. A suggestion for using powerful and informative test of normality. The American Statistician. 1990;44(4):316-21.
32. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nat Med. 2004;10(8):789-99.
33. Crespo P, Leon J. Ras proteins in the control of the cell cycle and cell differentiation. Cellular and Molecular Life Sciences. 2000;57(11):1613-36.
34. Tuveson DA, Shaw AT, Willis NA, Silver DP, Jackson EL, Chang S, et al. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell. 2004;5(4):375-87.
35. Valtorta E, Misale S, Sartore-Bianchi A, Nagtegaal ID, Paraf F, Lauricella C, et al. KRAS gene amplification in colorectal cancer and impact on response to EGFR-targeted therapy. International Journal of Cancer. 2013;133(5):1259-65.
36. Serebriiskii IG, Connelly C, Frampton G, Newberg J, Cooke M, Miller V, et al. Comprehensive characterization of RAS mutations in colon and rectal cancers in old and young patients. Nat Commun. 2019;10(1):3722.
37. Guo F, Gong H, Zhao H, Chen J, Zhang Y, Zhang L, et al. Mutation status and prognostic values of KRAS, NRAS, BRAF and PIK3CA in 353 Chinese colorectal cancer patients. Sci Rep. 2018;8(1):6076.
38. Chuang SC, Huang CW, Chen YT, Ma CJ, Tsai HL, Chang TK, et al. Effect of KRAS and NRAS mutations on the prognosis of patients with synchronous metastatic colorectal cancer presenting with liver-only and lung-only metastases. Oncol Lett. 2020;20(3):2119-30.
39. Liu P, Wang Y, Li X. Targeting the untargetable KRAS in cancer therapy. Acta Pharm Sin B. 2019;9(5):871-9.

40. Piton N, Lonchamp E, Nowak F, Sabourin JC, group K. Real-Life Distribution of KRAS and NRAS Mutations in Metastatic Colorectal Carcinoma from French Routine Genotyping. Cancer Epidemiol Biomarkers Prev. 2015;24(9):1416-8.
41. van Wyk HC, Roseweir A, Alexander P, Park JH, Horgan PG, McMillan DC, et al. The Relationship Between Tumor Budding, Tumor Microenvironment, and Survival in Patients with Primary Operable Colorectal Cancer. Ann Surg Oncol. 2019;26(13):4397-404.
42. Ozer SP, Barut SG, Ozer B, Catal O, Sit M. The relationship between tumor budding and survival in colorectal carcinomas. Rev Assoc Med Bras (1992). 2019;65(12):1442-7.
43. Dawson H, Lugli A. Molecular and pathogenetic aspects of tumor budding in colorectal cancer. Front Med (Lausanne). 2015;2:11.
44. Trinh A, Ladrach C, Dawson HE, Ten Hoorn S, Kuppen PJK, Reimers MS, et al. Tumour budding is associated with the mesenchymal colon cancer subtype and RAS/RAF mutations: a study of 1320 colorectal cancers with Consensus Molecular Subgroup (CMS) data. Br J Cancer. 2018;119(10):1244-51.
45. Maffeis V, Nicole L, Cappellesso R. RAS, Cellular Plasticity, and Tumor Budding in Colorectal Cancer. Front Oncol. 2019;9:1255.
46. Rimbert J, Tachon G, Junca A, Villalva C, Karayan-Tapon L, Tougeron D. Association between clinicopathological characteristics and RAS mutation in colorectal cancer. Mod Pathol. 2018;31(3):517-26.
47. Rosty C, Young JP, Walsh MD, Clendenning M, Walters RJ, Pearson S, et al. Colorectal carcinomas with KRAS mutation are associated with distinctive morphological and molecular features. Mod Pathol. 2013;26(6):825-34.
48. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012;486(7404):532-6.
49. Elsabah MT, Adel I. Immunohistochemical assay for detection of K-ras protein expression in metastatic colorectal cancer. J Egypt Natl Canc Inst. 2013;25(1):51-6.
50. Piton N, Borrini F, Bolognese A, Lamy A, Sabourin JC. KRAS and BRAF Mutation Detection: Is Immunohistochemistry a Possible Alternative to Molecular Biology in Colorectal Cancer? Gastroenterol Res Pract. 2015;2015:753903.
51. Dhawan A, Scott JG, Harris AL, Buffa FM. Pan-cancer characterisation of microRNA across cancer hallmarks reveals microRNA-mediated downregulation of tumour suppressors. Nature Communications. 2018;9(1):5228.
52. Niveditha D, Jasoria M, Narayan J, Majumder S, Mukherjee S, Chowdhury R, et al. Common and Unique microRNAs in Multiple Carcinomas Regulate Similar Network of Pathways to Mediate Cancer Progression. Sci Rep. 2020;10(1):2331.
53. Jevsinek Skok D, Hauptman N, Bostjancic E, Zidar N. The integrative knowledge base for miRNA-mRNA expression in colorectal cancer. Sci Rep. 2019;9(1):18065.
54. Fadaka AO, Pretorius A, Klein A. MicroRNA Assisted Gene Regulation in Colorectal Cancer. Int J Mol Sci. 2019;20(19).
55. Moszynska A, Gebert M, Collawn JF, Bartoszewski R. SNPs in microRNA target sites and their potential role in human disease. Open Biol. 2017;7(4).
56. Chin LJ, Ratner E, Leng SG, Zhai RH, Nallur S, Babar I, et al. A SNP in a let-7 microRNA Complementary Site in the KRAS 3 ' Untranslated Region Increases Non-Small Cell Lung Cancer Risk. Cancer Research. 2008;68(20):8535-40.
57. Chen M, Liu YY, Zheng MQ, Wang XL, Gao XH, Chen L, et al. microRNA-544 promoted human osteosarcoma cell proliferation by downregulating AXIN2 expression. Oncol Lett. 2018;15(5):7076-82.
58. Smits KM, Paranjape T, Nallur S, Wouters KA, Weijenberg MP, Schouten LJ, et al. A let-7 microRNA SNP in the KRAS 3'UTR is prognostic in early-stage colorectal cancer. Clinical Cancer Research. 2011;17(24):7723-31.

59. Christensen BC, Moyer BJ, Avissar M, Ouellet LG, Plaza SL, McClean MD, et al. A let-7 microRNA-binding site polymorphism in the KRAS 3' UTR is associated with reduced survival in oral cancers. Carcinogenesis. 2009;30(6):1003-7.
60. Liang D, Meyer L, Chang DW, Lin J, Pu X, Ye Y, et al. Genetic variants in MicroRNA biosynthesis pathways and binding sites modify ovarian cancer risk, survival, and treatment response. Cancer Res. 2010;70(23):9765-76.
61. Ganzinelli M, Rulli E, Caiola E, Garassino MC, Broggini M, Copreni E, et al. Role of KRAS-LCS6 polymorphism in advanced NSCLC patients treated with erlotinib or docetaxel in second line treatment (TAILOR). Sci Rep. 2015;5:16331.
62. Nelson HH, Christensen BC, Plaza SL, Wiencke JK, Marsit CJ, Kelsey KT. KRAS mutation, KRAS-LCS6 polymorphism, and non-small cell lung cancer. Lung Cancer. 2010;69(1):51-3.
63. Uvirova M, Simova J, Kubova B, Dvorackova N, Tomaskova H, Sedivcova M, et al. Comparison of the prevalence of KRAS-LCS6 polymorphism (rs61764370) within different tumour types (colorectal, breast, non-small cell lung cancer and brain tumours). A study of the Czech population. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2015;159(3):466-71.

FIGURES:

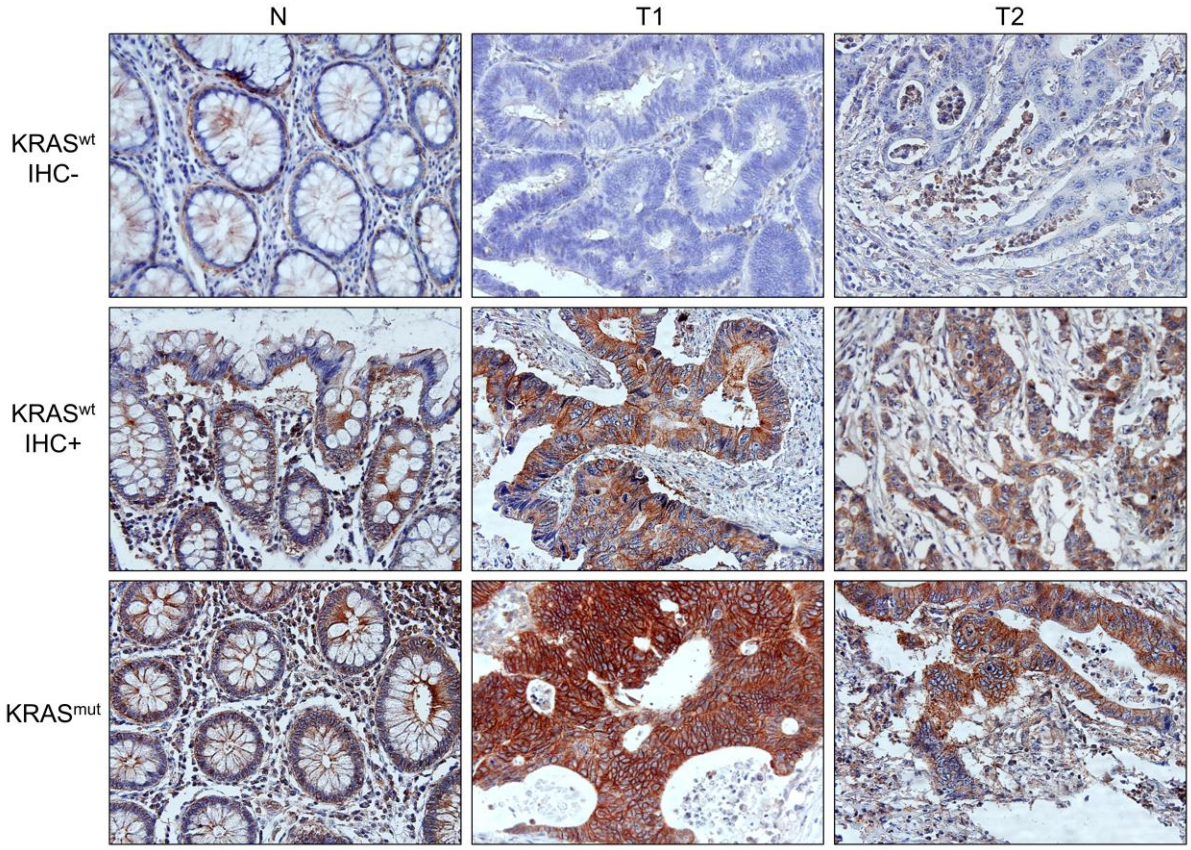

Figure 1. Immunohistochemical expression of KRAS protein in colorectal cancer. Representative images of KRAS protein expression in adjacent normal tissue (N), tumor center (T1), and invasive tumor front (T2) in *KRAS* wild-type ($KRAS^{wt}$) tumor samples either expressing KRAS protein (IHC+) or not (IHC-) as well as KRAS protein expression in *KRAS*-mutated tumor ($KRAS^{mut}$) samples. N, T1, and T2 samples were all taken from the same individual (magnification, x400).

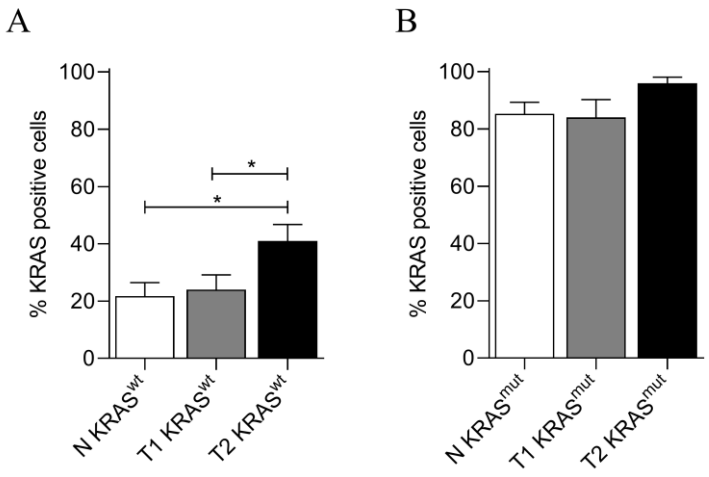

Figure 2. Quantitative assessment of KRAS positive cells in colorectal cancer. Percentage of immunohistochemically positive KRAS cells in (A) KRAS^{wt} and (B) KRAS^{mut} samples from adjacent normal tissue (N), tumor center (T1), and invasive tumor front (T2). All data are presented as means ± SEM and were analyzed by one-way ANOVA followed by Bonferroni's test ($p^{*} < 0.05$).

Figure 3. MicroRNAs and the sequence of the KRAS mRNA 3′-UTR. Schematic representation of miR-544a-3p and let-7a-5p predicted binding sites in KRAS 3′-UTR (marked red) as well as the location of the rs61764370 and rs10771184 polymorphisms (marked blue) in the 3′-UTR of KRAS

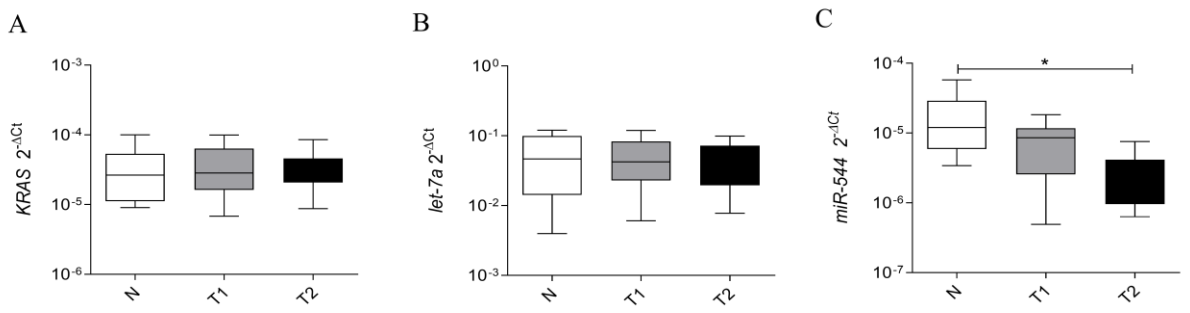

Figure 4. Expression of *KRAS* mRNA, *let-7a-5p,* and *miR-544a-3p* miRNA in KRAS wild-type colorectal cancer. Box plots show the expression of (A) *KRAS* mRNA, (B) *let-7a* and (C) *miR-544a-3p* in samples of adjacent normal tissue (N), tumor center (T1), and invasive tumor front (T2) of *KRAS* wild-type tumors. Data are presented as box and whisker plots (5-95 percentiles) and were analyzed by one-way ANOVA, followed by the Bonferroni's test (p*< 0.05).

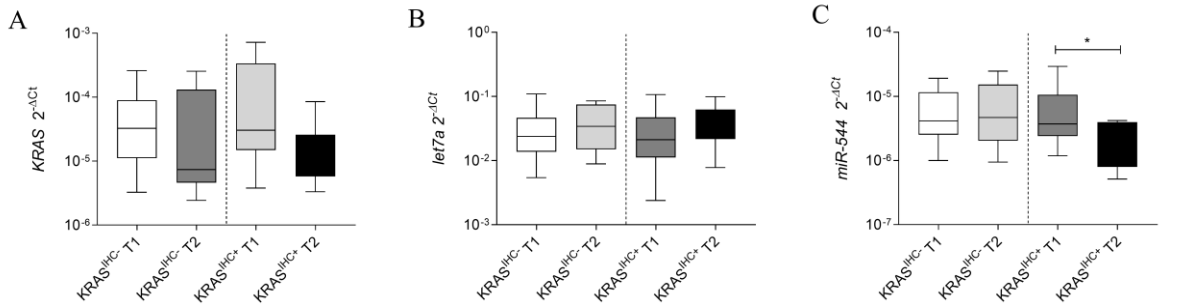

Figure 5. Differences in the expression of *KRAS* mRNA, *let-7a* and *miR-544a-3p* miRNA in KRAS protein-positive or protein-negative *KRAS* wild-type colorectal cancer. Box plots show the expression of (A) *KRAS* mRNA, (B) *let-7a* and, (C) *miR-544a-3p* in tumor center (T1) and invasive tumor front (T2) samples of *KRAS* wild-type colon carcinoma either expressing KRAS protein (IHC+) or not (IHC-). Data are presented as box and whisker plots (5-95 percentiles) and were analyzed using Student's t-test (p*< 0.05).

Table 1. Clinicopathological characteristics of 110 patients with sporadic adenocarcinoma stratified by KRAS mutation status

Characteristic	KRAS codon 12 or 13 mutation		p
	Negative (%)	Positive (%)	
	67 (60.9)	43 (39.1)	
Age			
< 70 years	47 (63.5)	27 (36.5)	0.422
≥ 70 years	20 (55.6)	16 (44.4)	
Gender			
Male	38 (57.6)	28 (42.4)	0.380
Female	29 (65.9)	15 (34.1)	
Tumor size			
≤ 5 cm	36 (55.4)	29 (44.6)	0.154
> 5 cm	31 (68.9)	14 (31.1)	
Histological grade			
Well (1)	24 (55.8)	19 (44.1)	0.474
Moderate (2)	36 (66.7)	18 (33.3)	
Poor (3)	7 (53.8)	6 (46.2)	
Dukes' stage			
A	4 (57.1)	3 (42.9)	
B	20 (74.1)	7 (25.9)	0.332
C	29 (60.4)	19 (39.6)	
D	14 (50.0)	14 (50.0)	
Tumor location			
Right-side	16 (51.6)	15 (48.4)	0.211
Left-side	51 (64.6)	28 (35.4)	
Tumor budding			
Low (1)	37 (68.5)	17 (31.5)	**0.038**
Moderate (2)	17 (68.0)	8 (32.0)	
High (3)	13 (41.9)	18 (58.1)	

Table 2. KRAS immunohistochemical expression intensity in N, T1, and T2 samples from wild-type or mutated KRAS tumors

	KRAS immunohistochemical score (+)					
KRAS status	0	1	2	3		p
Wild-type						
N	10 (55.6)	6 (33.3)	2 (11.1)	0 (0)		
T1	27 (64.3)	5 (11.9)	9 (21.4)	1 (2.4)	[a]0,818	[c]**0,030**
T2	17 (40.5)	8 (19.0)	16 (38.1)	1 (2.4)	[b]**0,039**	
Mutated						
N	1 (6.7)	8 (53.3)	5 (33.3)	1 (6.7)		
T1	0 (0)	6 (40.0)	5 (33.3)	4 (26.7)	[d]0,776	[f]0,076
T2	0 (0)	3 (20.0)	7 (46.7)	5 (33.3)	[e]**0,003**	

p values were obtained by Fisher's exact test. N, adjacent normal tissue; T1 tumor center; T2 invasive tumor front; KRAS, v-Ki-ras2-Kirsten rat sarcoma viral oncogene homolog; letter marks the difference between [a]$KRAS^{wt}$ N and T1, [b]$KRAS^{wt}$ N and T2, [c]$KRAS^{wt}$ T1 and T2; [d]$KRAS^{mut}$ N and T1, [e]$KRAS^{mut}$ N and T2, [f]$KRAS^{mut}$ T1 and T2

Table 3. Clinicopathological characteristics of 50 patients with wild-type *KRAS* sporadic colon cancer stratified by KRAS protein expression

Characteristic	KRAS protein		p
	Negative (%)	Positive (%)	
	19 (38.0)	31 (62.0)	
Age			
< 70 years	15 (40.5)	22 (59.5)	0.532
≥ 70 years	4 (30.8)	9 (69.2)	
Gender			
Male	10 (34.5)	19 (65.5)	0.546
Female	9 (42.9)	12 (57.1)	
Tumor size			
≤ 5 cm	13 (43.3)	17 (56.7)	0.341
> 5 cm	6 (30.0)	14 (70.0)	
Histological grade			
Well (1)	9 (45.0)	11 (55.0)	0.707
Moderate (2)	9 (33.3)	18 (66.7)	
Poor (3)	1 (33.3)	2 (66.7)	
Dukes' stage			
A	2 (50.0)	2 (50.0)	
B	2 (13.3)	13 (86.7)	0.131
C	11 (50.0)	11 (50.0)	
D	4 (44.4)	5 (55.6)	
Tumor location			
Right-side	3 (37.5)	5 (62.5)	0.974
Left-side	16 (38.1)	26 (61.9)	

Table 4. Wild-type KRAS sporadic colon cancer tumors stratified by KRAS protein expression and KRAS polymorphisms in miRNA binding sites

KRAS polymorphism (miRNA) Genotypes/Alleles	KRAS protein		p
	Negative (%) 19 (38.0)	Positive (%) 31 (62.0)	
KRAS rs61764370 T/G, LCS6 (*let-7a-5p*)			
TT	19 (100.0)	25 (80.6)	0.123
TG	0 (0.0)	5 (16.1)	
GG	0 (0.0)	1 (3.3)	
TT	19 (100.0)	25 (80.6)	**0.040**
TG + GG	0 (0.0)	6 (19.4)	
T	38 (100.0)	55 (88.7)	**0.031**
G	0 (0.0)	7 (11.3)	
KRAS rs10771184 A/T (*miR-544a-3p*)			
AA	3 (15.8)	9 (29.0)	
AT	13 (68.4)	13 (42.0)	0.190
TT	3 (15.8)	9 (29.0)	
AA	3 (15.8)	9 (29.0)	0.287
AT + TT	16 (84.2)	22 (71.0)	
A	19 (50.0)	31 (50.0)	1.00
T	19 (50.0)	31 (50.0)	