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IS THERE ANY ASSOCIATION BETWEEN TACSTD2, KIAA1253, KU70 AND MUTANT KRAS GENE EXPRESSION AND CLINICAL-PATHOLOGICAL FEATURES OF COLORECTAL CANCER?

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***Aim*: To determine the quantitative gene expression of *KRAS* codon 12 mutant, *TACSTD2*, *Ku70* and *SERIN1* in samples of tumor tissue and to relate them with clinical-pathological characteristics of colorectal cancer. *Methods:* Samples of tumor and normal tissue of patients surgically treated for colorectal cancer between July 2005 and July 2009 were stored in a tissue bank. These samples were studied with the technique of real-time polymerase chain reaction in respect to expression of the following genes: *KRAS* codon 12 mutation, *TACSTD2*, *Ku70*, and *SERIN1*. *Results:* Tumor samples of 37 patients were studied. The mean age was 65.5 years. Twenty one patients (56.8%) were male. Nine patients (24.3%) were classified as TNM stage I, 11 patients (29.8%) as TNM stage II, eight patients (21.6%) as TNM stage III and nine patients (24.3%) as TNM stage IV. The *Ku70* expression in poorly-differentiated tumors is significantly higher than in well and moderately-differentiated tumors (2.76 vs. 1.13; $p < 0.05$). *SERIN1*, *TACSTD2* and *KRAS* codon 12 mutation are not associated with clinical-pathological characteristics of colorectal cancer. *Conclusion: Ku70* expression in poorly-differentiated tumors is significantly higher than in well and moderately-differentiated colorectal tumors.**

***Key Words*: gene expression, neoplasm staging, polymerase chain reaction, intestine, molecular biology.**

Genetic disorders play a key role in the colorectal carcinogenesis, either in its initiation or its progression [1]. During the last years experts in colorectal cancer (CRC) have focused its attention mainly over the *KRAS* gene. *KRAS* mutations are observed in around 40% of CRC [2–5]. About 90% of these mutations occur in the codons 12 (70–80%) and 13 (20–30%) [2, 6, 7]. It has been demonstrated that mutations on codon 12 are associated with a poorer prognosis of CRC [3]. For this reason, many authors currently are looking for new genes potentially associated to the clinical-pathological features and the prognosis of patients with CRC.

The *tumor-associated calcium signal transducer* (*TACSTD2*), also known as *TROP2*, is located on chromosome 1 (1p32–1p31) [5]. *TACSTD2* overexpression has been observed in most human carcinomas and proposed as a possible stimulus for growth and tumor development [8–14]. Its overexpression has been associated with decreased overall survival, increased rate of CRC-related-death, and higher risk of liver metastasis [8, 15, 16]. The Ku70 protein is a homonymous polypeptide of 70 kDa. It is an antiapoptotic protein that plays an essential role in the repair of DNA double strand break damage induced by ionizing radiation in mammalian cells [17–19]. It was demonstrated that *Ku70* expression is associated with decreased disease-free-survival in CRC and with impaired response to radiotherapy in rectal cancer patients [20–21]. The *SERIN1* expression, not widely studied yet, seems also related to the prognosis of CRC patients, specifically with the overall survival at 36 months [22]. The aim of this study is to evaluate the association between *KRAS* codon 12 mutation, *TACSTD2*, *Ku70* and *SERIN1* expression, and clinical-pathological characteristics with prognostic relevance.

MATERIALS AND METHODS

Population and samples. This is a cross-sectional study which enrolled patients with CRC who were surgically treated at the Division of Colorectal Surgery of Hospital São Lucas, Pontificia Catholic University of Rio Grande do Sul, between July 2005 and July 2009. Samples of 1 cm^2 were taken from the center of the resected tumor, immediately frozen in liquid nitrogen, and subsequently stored in a tissue bank at — 80 °C as described previously [23–25]. Patients who underwent neoadjuvant radiochemotherapy, with familial adenomatous polyposis, hereditary nonpolyposis CRC, inflammatory bowel disease, synchronic or previous CRCs, any synchronic or previous cancer, incomplete data or with no expression of the positive control gene (β*2-microglobulin*) were excluded. All patients signed an informed consent form for the collection, storage and studying of their samples. The study protocol was approved by the local ethics committee.

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Abbreviations used: CRC – colorectal cancer; CEA – carcinoembryonic antigen; PCR – polymerase chain reaction; AJCC – American Joint Committee on Cancer

Clinical staging and pathological examination. Preoperative oncological workup included colonoscopy, computerized tomography and/or ultrasonography of the abdomen, and chest radiography and/or computerized tomography. Right and left-sided cancers were defined as cancer located respectively proximal and distal to the splenic flexure. Rectal cancer was defined as that situated under the sacral promontory. Pathological examinations were performed by the same pathologist according to the International Classification of Diseases for Oncology. Gene expression was compared to the following clinical-pathological features: age, gender, race, preoperative measurement of the carcinoembryonic antigen (CEA), tumor location, TNM classification, American Joint Committee on Cancer (AJCC) stage, liver and lung metastasis, tumor grade, vascular invasion, and mucus production [26].

Technique of molecular analysis. The RNA extractions were performed with Trizol (Trizol® Reagent, Invitrogen, USA) from a sample of 100 mg of tumor tissue, according to the manufacturer's instructions. The quantity and quality of RNA were determined with spectrophotometry (Gene Quant®, Pharmacia Biotech, USA), from duplicate aliquots of 1 ml of solution. The synthesis of complementary DNA (cDNA) was performed from 2 mg of the total RNA through polymerase chain reaction (PCR) with reverse transcription (SuperScript First-Strand Synthesis System®, Invitrogen, USA). The real-time PCRs were performed with the DNA Engine Opticon 2 Real-Time PCR System® (Bio-Rad, USA). The final volume per reaction was 25 µl, containing 2 µl of cDNA diluted 10 times, 0.1 µM of primer sense and antisense, 12.5 µl of Platinun-SybrGreen qPCR Supermix-UDG® and ultrapure water q.s.p. β2-microglobulin served as a positive control in each experiment, while PCR reagents without template were run in parallel as no template controls. The conditions of the reactions were: 94 °C (2 min), 94 °C (50 s), X °C (45 s), 72 °C (45 s), followed by 45 cycles with a final extension of 2 min at 72 °C and melting curve of 56 to 96 °C (increasing by 0.5 °C each 10 s). Primers sequences were *TACSTD2* (sense: TGACCTCCAAGTGTCTGCTG/ antisense: GTCGTAGAGGCCATCGTTGT), *Ku70* (sense: CCACAGGAAGAAGAGTTGGA / antisense: CTGCTCTGGAGTTGCCATGA), *SERIN1* (sense: TGATGGATCACTGGAGGATG / antisense: AGCATGAAGTGAAAGAAGGA) and *KRAS* codon 12 (sense: GACTGAATATAAACTTGTGG / antisense: CCAGGTCCTGGTAAGAAACT). The annealing temperature was 57 °C for all genes, except for *SERIN1* (54 °C).

Statistical analysis. The *SPSS*® software, version 15.0, was used for statistical analysis. Mann — Whitney and the Kruskal — Wallis tests were used to compare categorical and quantitative variables, respectively. Pearson's correlation coefficient was used to compare non-parametric quantitative variables. The p values < 0.05 were considered to indicate statistical significance.

RESULTS AND DISCUSSION

In molecular study samples from 37 patients were analyzed. The exclusion criterion adopted aimed to select a homogeneous sample, composed exclusively of subjects with sporadic CRC. Once it is known that the chemoradiotherapy reduces the amount of tumor cells and interferes in the performance of the PCR assay, we decided to exclude patients who underwent preoperative chemoradiotherapy. That is the reason why the sample studied showed fewer rectal tumors (18.9%) than usually described in other publications [6]. The sample fixation in formalin can chemically modified the DNA structure and interfere in the reaction efficiency. For this reason, we used fresh frozen samples instead of formalin-fixed paraffin-embedded tumor samples [27]. Patients' demographic and clinical data and tumor pathological features are summarized in Table 1. Two patients (5.4%) had peritoneal implants diagnosed during the surgery, while one patient (2.7%) had lung metastasis discovered during the clinical staging.

Table 1. Clinical-pathological characteristics

	N = 37 (%)
Gender	
• Male	21(56.8)
• Female	16 (43.2)
Age (years) [a]	65.5 (12.1)
Race	
• Caucasian	34 (91.9)
• African-American	1 (2.7)
• Asian	2 (5.4)
Preoperative CEA (ng/mL) [b]	2.5 (1.0; 5.4)
Tumor location	
• Right colon	12 (32.4)
• Left colon	18 (48.7)
• Rectum	7 (18.9)
Category T (TNM)	
• T1	7 (18.9)
• T2	2 (5.4)
• T3	27 (73.0)
• T4	1 (2.7)
Category N (TNM)	
• N0	20 (54.1)
• N1	13 (35.1)
• N2	4 (10.8)
Category M (TNM)	
• M0	9 (24.4)
• M1	28 (75.6)
Liver metastasis	
• Absence	31 (83.6)
• Presence	6 (16.2)
AJCC stage	
• I	7 (24.3)
• II	11 (29.8)
• III	8 (21.6)
• IV	9 (24.3)
Tumor grade	
• G1 or G2	35 (94.6)
• G3	2 (5.4)
Vascular invasion	
• Presence	7 (18.9)
• Absence	30 (81.1)
Mucus production	
• Presence	5 (13.5)
• Absence	32 (86.5)

Note: G1: well-differentiated; G2: moderately-differentiated; G3: poorly-differentiated; [a]mean (standard deviation) value expressed; [b]median (25% and 75% quartile) value expressed.

Male patients with CRC showed a higher *TACSTD2* and *SERIN1* expression than women (*TACSTD2* = 2.04 *vs.* 0.56, $p = 0.381$; and *SERIN1* = 1.79 *vs.* 1.59, $p = 0.415$). This tendency is opposite to that observed in respect to *Ku70* expression (male: 1.02 *vs.* female: 1.18, $p = 0.307$) and *KRAS* codon 12 mutation (male: 0.42 *vs.*

female: 0.48, $p = 0.817$). Despite these facts the associations between gene expressions and gender were not statistically significant. *TACSTD2*, *SERIN1* and *KRAS* codon 12 mutation expressions were inversely proportional to patient's age ($r_s = -0.217$, $r_s = -0.193$ and $r_s = -0.146$, respectively) and were also not statistically significant ($p = 0.332$, $p = 0.306$ and $p = 0.460$ respectively).

The *Ku70* quantitative expression was directly proportional to patient's age, but with a low grade of correlation ($r_s = 0.041$), and no statistical significance ($p = 0.824$). Fig. illustrates *Ku70* real-time PCR results.

Distal tumors presented higher expression of *TACSTD2* (rectum = 4.99 *vs.* left colon = 1.67 *vs.* right colon = 0.57, $p = 0.236$), while proximal tumors tend to present higher expression of *Ku70* (right colon = 1.85 *vs.* left colon = 1.13 *vs.* rectum = 0.78, $p = 0.413$). *SERIN1* and *KRAS* codon 12 mutation did not show a pattern of quantitative gene expression according to tumor localization (right colon = 1.88 and 0.48 *vs.* left colon = 2.78 and 0.22 *vs.* rectum = 2.00 and 0.69, $p = 0.747$ and 0.337, respectively). Gene expressions and CEA preoperative measurement showed low correlation and no statistically significant association. Data regarding the correlation between gene expression and quantitative variables, age and CEA, are shown in Table 2.

Table 2. Correlation between quantitative variables and gene expressions

	TACSTD2		KU70		SERIN1		KRAS codon 12	
	r_s	p	r_s	p	r_s	p	r_s	p
Age	-0.217	0.332	0.041	0.824	-0.193	0.306	-0.146	0.460
CEA	0.088	0.729	-0.108	0.607	0.210	0.337	-0.351	0.118

TACSTD2 and *Ku70* quantitative expression were lower in tumors with vascular invasion on histopathology examination (0.04 *vs.* 0.95, $p = 0.557$; and 0.72 *vs.* 1.18; $p = 0.381$, respectively). This finding is contrary to that observed in respect to *SERIN1* (4.90 *vs.* 1.37, $p = 0.649$) and *KRAS* codon 12 mutation (0.48 *vs.* 0.42, $p = 0.874$) expressions. The *Ku70* expression in poorly-differentiated tumors is statistically higher than in well and moderately-differentiated tumors (2.76 *vs.* 1.13, $p = 0.030$). No association was observed between tumor grade and *TACSTD2*, *SERIN1* and *KRAS* codon 12 mutation. Lower quantitative expression of all genes was observed among tumors with intra or extra-cellular mucus production (*TACSTD2* = 0.33 *vs.* 0.95, $p = 0.312$; *Ku70* = 0.90 *vs.* 1.17, $p = 0.392$; *SERIN1* = 1.29 *vs.* 1.92, $p = 0{,}839$): *KRAS* codon 12 mutation = 0.33 *vs.* 0.45, $p = 0.762$).

With regard to category T, tumors with deeper invasion of the colorectal wall revealed higher *TACSTD2* expression (T1 = 0.48 *vs.* T2 = 1.16 *vs.* T3 = 2.47, $p = 0.409$). *KU70*, *SERIN1* and mutant *KRAS* did not show any expression trend. The only patient with T4 tumor was excluded from the analysis. *TACSTD2, Ku70* and *SERIN1* quantitative expression showed no statistically significant association with category N (*TACSTD2:* N0 = 0.79 *vs.* N1 = 4.21 *vs.* N2 = 0.22, $p = 0.536$; Ku70: N0 = 1.18 *vs.* N1 = 0.86 *vs.* N2 = 1.36, $p = 0.567$; *SERIN1*: N0 = 2.03 *vs.* N1 = 1.45 *vs.* N2 = 0.46, $p = 0.606$; *KRAS* codon 12 mutation: N0 = 0.45 *vs.* N1 = 1.32 *vs.* N2 = 0.01, $p = 0.254$). Data concerning the occurrence of systemic metastasis (category M) and TNM staging were not statistically associated with *TACSTD2, Ku70* and *SERIN1* expressions, and are presented in table 3. Patients with liver metastasis presented higher gene expression levels (*TACSTD2* = 8.45 *vs.* 0.63, $p = 0.693$; *Ku70* = 1.89 *vs.* 1.13, $p = 0.256$; *SERIN1* = 1.88 *vs.* 1.69, $p = 0.219$). The exception was mutant *KRAS* (0.02 *vs.* 0.54, $p = 0.264$). These data and the analysis of all the other categorical variables are represented in Table 3.

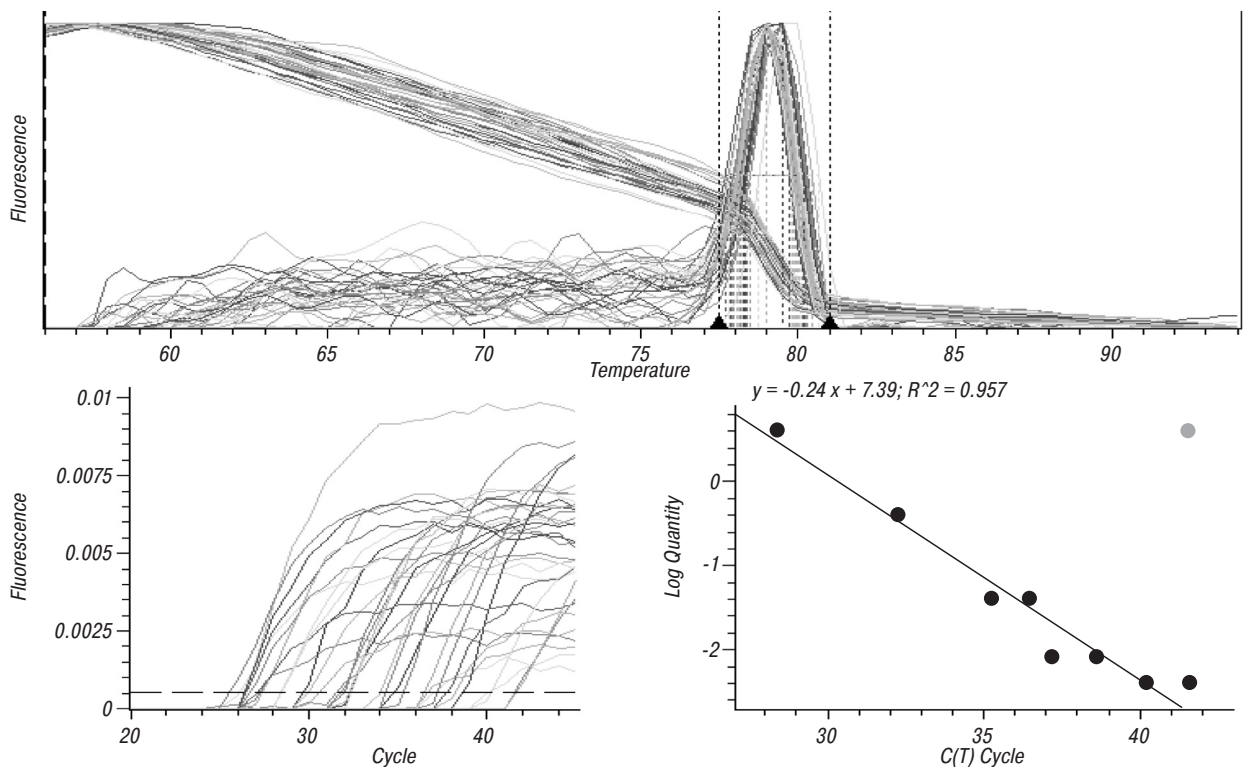

Figure. Ku70 real-time PCR: *a*, melting curve; *b*, amplification curve; *c*, linear regression of the results

Table 3. Relation between categorical variables and TACSTD2, Ku70, SERIN1 and KRAS codon12 mutation expressions

	TACSTD2	KU70	SERIN1	KRAS codon 12
Gender				
• Male	2.04 (0.37–8.99)	1.02 (0.70–1.55)	1.79 (0.68–6.87)	0.42 (0.04–1.82)
• Female	0.56 (0.17–4.71)	1.18 (1.03–26.47)	1.59 (0.40–3.70)	0.48 (0.02–2.98)
Tumor site [b]				
• Right colon	0.57 (0.08–0.71)	1.85 (0.72–2.26)	1.88 (0.74–2.12)	0.48 (0.22–1.92)
• Left colon	1.67 (0.07–13.46)	1.13 (0.74–1.25)	2.78 (1.08–8.81)	0.22 (0.02–2.40)
• Rectum	4.99 (4.21–5.77)	0.78 (0.23–1.71)	2.00 (0.62–4.24)	0.69 (0.02–8.17)
Vascular invasion [a]				
• Presence	0.04 (0.03–10.0)	0.72 (0.22–2.99)	4.90 (0.26–8.27)	0.48 (0.04–1.95)
• Absence	0.95 (0.41–5.77)	1.18 (0.82–1.75)	1.37 (0.51–5.87)	0.42 (0.01–2.98)
Tumor grade [a]				
• G1 or G2	1.31 (0.25–6.59)	1.13 (0.71–1.59)	1.45 (0.49–5.87)	0.38 (0.02–1.82)
• G3	0.59 (0.59–0.59)	2.76 (1.19–4.33)(c)	1.17 (0.46–1.88)	10.0 (1.90–18.12)
Mucus production				
• Presence	0.33 (0.04–0.63)	0.90 (0.33–1.64)	1.29 (0.57–7.54)	0.33 (0.06–0.61)
• Absence	0.95 (0.29–6.31)	1.17 (0.49–2.66)	1.92 (0.91–4.44)	0.45 (0.02–1.94)
Category T (TNM) [d]				
• T1	0.48 (0.24–1.11)	1.17 (0.93–1.51)	1.93 (1.09–4.23)	1.94 (0.09–2.84)
• T2	1.16 (1.10–2.42)	1.71 (1.21–2.21)	15.38 (0.73–30.0)	0.20 (0.02–0.38)
• T3	2.47 (0.31–10.92)	1.11 (0.72–1.85)	1.45 (0.47–5.26)	0.45 (0.05–2.17)
Category N (TNM) [b]				
• N0	0.79 (0.43–3.60)	1.18 (0.91–2.12)	2.03 (0.54–5.46)	0.45 (0.09–1.96)
• N1	4.21 (0.19–14.89)	0.86 (0.34–1.53)	1.45 (0.58–6.71)	1.32 (0.03–2.44)
• N2	0.22 (0.22 – 0.22)	1.36 (0.66–2.62)	0.46 (0.32–3.94)	0.01 (0.01–18.13)
Category M (TNM) [a]				
• M0	2.05 (0.49–6.59)	1.18 (0.77–1.94)	1.69 (0.56–4.96)	0.43 (0.04–1.95)
• M1	0.34 (0.13–8.46)	0.92 (0.59–2.26)	1.88 (0.46–6.71)	1.68 (0.01–2.98)
Liver metastasis				
• Yes	8.45 (0.58–16.32)	1.89 (0.72–3.99)	1.88 (1.00–3.93)	0.06 (0.01–1.89)
• No	0.63 (0.08–4.99)	1.13 (0.40–1.85)	1.69 (0.67–4.33)	0.54 (0.12–1.96)
AJCC stage [b]				
• I	0.48 (0.10–1.68)	1.20 (1.17–1.98)	1.93 (0.91–7.04)	1.16 (0.06–2.40)
• II	3.99 (0.79–8.47)	1.15 (0.75–2.31)	2.13 (0.37–5.87)	0.42 (0.09–0.61)
• III	9.62 (4.60–2.94)	0.98 (0.15–1.89)	1.37 (0.56–6.74)	0.05 (0.01–3.08)
• IV	0.34 (0.13–8.46)	0.92 (0.59–2.26)	1.88 (0.46–6.71)	1.68 (0.01–2.98)

Note: Gene expression presented through median and inter–quartile range (IQR1; IQR3); [a]Mann – Whitney test; [b]Kruskal–Wallis test; [c]$p < 0.05$; [d]one patient excluded.

Despite the great interesting of the international scientific community and the numerous publications about *KRAS* gene, testing for *KRAS* mutations still currently not standardized [6]. It is known however that real-time PCR is the test with the greatest sensitivity (96.5%) to detect *KRAS* mutations [28]. In agreement with Cejas *et al.* [29], we did not observed association between mutation in codon 12 and the clinical-pathological variables studied. Chang *et al.* [30] analyzed 228 cases of CRC with a multiplex PCR and did not report association between mutation in codon 12 and any clinical-pathological feature other than lymph nodes metastasis ($p = 0.048$). This finding however was not confirmed in our study.

TACSTD2, also known as *TROP2*, codifies a protein that promotes anchorage-independent growth and tumorigenesis [31, 32]. *TROP2* overexpression occurs mainly in rectal cancer and is associated with disease recurrence and increased cancer-related-deaths [9, 16]. Our findings support Ohmachi *et al.* [9], once both studies did not identify association between *TROP2* expression in tumor tissue and age, gender, tumor site, histological grade, vascular invasion and lymph node metastasis. Unlike this author, we did not observe association between *TROP2* expression and the occurrence of liver metastasis [9]. Our study analyzed tumors of the colon and rectum together and like Fang *et al.* [16] we did not identify association between the *TROP2* expression and the occurrence of liver metastasis.

Preoperative radiotherapy reduces the rate of local recurrence and improves the chance of survival in patients with resectable, advanced rectal cancer. Targeting the *Ku70* and/or *Ku80* could inhibit repair of the radiation-induced DNA double strand break damage, enhancing the radiation response of tumor cells [20, 21]. Our study was the first to describe the association between higher *Ku70* expression and low-differentiated (tumor grade G3) colorectal cancer ($p = 0.03$). An immunohistochemistry study of CRC patients, performed by Komuro *et al.* [20, 21], did not demonstrate association between the *Ku70* expression and clinical-pathological features, except the depth of tumor invasion. The explanation for this difference may be: (1) limitations of the real-time PCR and immunohistochemistry assay; (2) discrepancy between gene and protein expressions; or (3) compensatory gene expression in the homologous recombination pathway [21, 33].

The first citation in literature about SERIN1 derives from the publication of Eschrich *et al.* [22]. Using the technique of cDNA microarray, this author established a panel of 43 genes, including *SERIN1*, associated with an unfavorable prognosis of patients with CRC [22]. We did not observe association between *SERIN1* expression and clinical-pathological characteristics of CRC. Until the current date our report is the unique study concerning this topic.

Some potential limitations of our study require further discussion. First, the small number of patients

studied difficults the identification of any eventually statistically significant difference. Another limitation is that we investigate only one type of *KRAS* mutation, precluding us from generalizing our conclusions about other mutations of this gene. Finally, we analyzed colon and rectal tumors together, making difficult to compare our findings with those reported by some other authors.

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