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High frequency of NRAS mutations and RAS co-mutations in colorectal cancer from the Kurdistan region of Iraq: molecular and survival analysis

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Abstract

Mutations in *RAS* and *PI3K* pathway genes play a critical role in Colorectal Cancer (CRC) pathogenesis and therapy response, yet data from underrepresented populations remain limited. Molecular analysis was performed on 48 CRC cases from the Kurdistan region of Iraq to detect mutations in *KRAS* and *NRAS* (codons 12, 13, 59, 61, 117, and 146; exons 2–4), *BRAF* (codon 600; exon 15), *PIK3CA* (codons 542, 545, and 1047; exons 9 and 20), and *AKT1* (codon 17; exon 4), and to correlate findings with histopathological characteristics and survival outcomes. *NRAS* mutations were the most frequently detected alterations (52.08%), followed by *KRAS* (47.92%) and *PIK3CA* (16.67%). A high frequency of co-mutations, particularly involving *KRAS* and *NRAS*, was observed, substantially exceeding frequencies reported in international cohorts; possible contributing factors including intratumoral heterogeneity and assay sensitivity are discussed. Tumor-associated inflammatory response and lymph node status demonstrated significant associations with patient survival. These findings from a small patient cohort suggest the need for further investigation of regional CRC molecular profiles using larger cohorts and orthogonal validation methods.

Introduction

Colorectal Cancer (CRC) is a major global public health challenge, ranking as the third most commonly diagnosed cancer and the second leading cause of cancer-related mortality worldwide.¹ According to the World Health Organization (WHO), more than 1.9 million new CRC cases and over 930,000 deaths were reported globally in 2020.² The global burden of CRC is projected to increase substantially by 2040, reaching approximately 3.2 million new cases (a 63% increase) and 1.6 million deaths (a 73% increase) annually.³

Although advances in screening, diagnosis, and treatment have contributed to declining mortality rates in high-income countries, particularly among older populations, both incidence and mortality of CRC are rising in many low- and middle-income countries. These include Poland, Romania, China, Brazil, Colombia, and Iran.³⁻⁸ This increasing trend has been largely attributed to the adoption of Westernized lifestyles, characterized by unhealthy dietary patterns, physical inactivity, tobacco use, excessive alcohol consumption, and increasing obesity rates.^{5,7}

The prognosis, risk stratification, diagnostic evaluation, and therapeutic decision-making in CRC are influenced by a complex interaction of clinicopathological features, histological characteristics, Tumor–Node–Metastasis (TNM) staging, and molecular biomarkers.^{9,10} In recent years, increasing evidence has demonstrated that genetic and molecular biomarkers provide greater predictive and prognostic value than traditional clinicopathological parameters, particularly in guiding targeted therapies.^{11,12}

Among the most extensively studied molecular pathways in CRC is the Epidermal Growth Factor Receptor (EGFR) signaling pathway, along with its downstream effectors, including the *RAS* gene family (*KRAS* and *NRAS*) and other oncogenes such as *BRAF*, *PIK3CA*, and *AKT1*.^{8,13,14} Mutations in *KRAS*, *NRAS*, and *BRAF* have been strongly associated with resistance to anti-EGFR monoclonal antibody therapies, such as cetuximab, and with poor clinical outcomes.¹³ Similarly, *PIK3CA* mutations particularly in tumors with wild-type *KRAS* have been linked to unfavorable prognosis and therapeutic resistance.^{12,15}

At the regional level, data from the Duhok Health Directorate indicate that CRC represents a major cause of cancer-related mortality, ranking fourth in 2018 and 2020, third in 2019, and second in

2021. CRC accounted for 10.16% of cancer-related deaths in the province.¹⁶ Despite this increasing burden, comprehensive molecular profiling and population-based studies of CRC remain limited in this region, like many developing areas.

Comprehensive characterization of the molecular landscape of CRC, particularly RAS/PI3K pathway alterations, may improve diagnostic accuracy, refine prognostic assessments, and guide targeted therapies. Despite the rising burden of CRC in Duhok, population-based molecular studies remain limited, highlighting the need for deeper investigation.

Materials and Methods

Study design and patients

This cross-sectional study included 48 patients with histopathologically confirmed CRC diagnosed between January 2013 and December 2015. The study period was deliberately defined to ensure a minimum follow-up of five years for all patients while avoiding the confounding effects of the COVID-19 pandemic on cancer survival outcomes, treatment access, and mortality patterns, which emerged globally from 2020 onwards. Follow-up data were collected in 2021. Formalin-Fixed Paraffin-Embedded (FFPE) tumor tissue blocks were obtained from the Pathology Department, Central Laboratory, Azadi Teaching Hospital, Duhok Governorate, Kurdistan Region, Iraq. Relevant clinical and pathological data, including age, sex, clinical presentation, disease history, type of surgery, tumor characteristics, and number of excised lymph nodes, were retrieved from pathology records. Survival status was verified through direct contact with surviving patients, relatives of deceased patients, or the operating surgeons. Patients were stratified into three groups based on survival: <3 years, 3–5 years, and >5 years.

Inclusion and exclusion criteria

Patients were included if they had resected CRC with clear margins, at least 12 examined lymph nodes, complete pathology records, and verified survival data. Exclusion criteria were incomplete survival information, death unrelated to CRC, lack of excisional biopsy, or examination of fewer than 12 lymph nodes.

Histopathological examination

Hematoxylin and Eosin (H&E) stained slides were independently reviewed by two pathologists. Tumors were classified according to WHO criteria¹⁷ and graded/staged according to the guidelines of the American Joint Committee on Cancer (AJCC) and the American Cancer Society (ACS).¹⁸ Representative formalin-fixed, paraffin-embedded (FFPE) tissue blocks with adequate tumor content were selected for molecular analysis. Histopathological variables were assessed using standardized criteria.

Tumor-associated inflammatory reaction was evaluated semiquantitatively based on the density of inflammatory cell infiltrates at the tumor invasive margin and within the tumor stroma and was classified as mild (sparse inflammatory infiltrate predominantly at the tumor periphery), moderate (inflammatory infiltrate present at both the invasive margin and within the tumor stroma), or severe (dense, band-like inflammatory infiltrate at the invasive margin).

Desmoplastic reaction was graded according to the extent of stromal fibrosis surrounding tumor glands and classified as mild (minimal fibrous stroma with predominant tumor glands), moderate (intermediate fibrous stroma with identifiable tumor glands), or severe (extensive fibrous stroma with compressed tumor glands).

Tumor budding was assessed according to the International Tumor Budding Consensus Conference (ITBCC 2016) criteria by counting dedifferentiated single cells or clusters of up to four cells at the tumor invasive front within a standardized 0.785 mm² field. Tumor budding was categorized as low (0–4 buds), intermediate (5–9 buds), or high (≥ 10 buds).

Molecular analysis

DNA extraction

Genomic DNA was extracted from 8–10 μm sections of macrodissected FFPE tumor tissue using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Tissue sections were cut at 8–10 μm thickness from representative FFPE blocks selected by the reviewing pathologists. Macrodissection was performed under direct microscopic visualization to maximize tumor cell content and minimize contamination from adjacent normal or stromal tissue. Following extraction, DNA concentration and purity were assessed using the

NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Samples with A260/A280 absorbance ratios outside the accepted range of 1.7–2.0 or DNA concentrations below 2 ng/μl were excluded from further analysis. Extracted DNA was stored at -20°C until molecular analysis. To verify intra-assay consistency, three randomly selected samples were independently re-analyzed using the identical extraction and amplification protocol, yielding fully concordant results.

Real-Time Polymerase chain reaction (PCR) mutation detection and analysis

Mutation profiling was performed using the Colorectal Cancer Mutation Detection Panel (EntroGen Inc., Woodland Hills, CA, USA), a CE (Conformité Européenne)-marked multiplex real-time PCR assay with an analytical sensitivity of $\geq 1\text{--}5\%$ mutant allele frequency, validated for use with FFPE-derived DNA. The assay detects clinically relevant mutations in *KRAS* (codons 12, 13, 59, 61, 117, and 146; exons 2, 3, and 4), *NRAS* (codons 12, 13, 59, 61, 117, and 146; exons 2, 3, and 4), *BRAF* (codon 600; exon 15), *PIK3CA* (codons 542, 545, and 1047; exons 9 and 20), and *AKT1* (codon 17; exon 4). Primer and probe sequences are proprietary to the manufacturer and not publicly disclosed by EntroGen Inc.; full assay technical specifications are available in the manufacturer's product documentation. DNA input was standardized across all samples per manufacturer recommendations, and an endogenous internal control gene was included in each reaction to verify DNA integrity and amplification efficiency. PCR amplification was performed on the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using Sequence Detection Software (SDS) software. Mutations were called based on the presence of mutation-specific amplification curves crossing a predefined fluorescence threshold; samples in which the internal control failed to amplify within the defined threshold were excluded from mutation analysis.

Statistical Analysis

Demographic and clinicopathological data were summarized as mean $\pm$ standard deviation or frequency (%). Mutation frequencies were calculated relative to the total cohort (n=48). Survival curves were generated using the Kaplan–Meier method. Categorical variables were compared using Pearson's chi-square test or Fisher's exact test, as appropriate. Binary logistic regression was used to estimate odds ratios and 95% confidence intervals for short survival, defined as survival of <3

years. All tests were two-sided, and $p < 0.05$ was considered statistically significant. Because no adjustment for multiple comparisons was performed, the findings were considered exploratory. Analyses were conducted using JMP Pro, version 14.3.0 (SAS Institute Inc., Cary, NC, USA).

Results

Clinicopathological characteristics of patients

A total of 48 patients with CRC were included in the study, and their clinicopathological characteristics are summarized in Supplementary materials, Table 1. The mean age at diagnosis was 58.40 ± 16.98 years (range: 19–84). Men comprised 58.33% and women 41.67% of the study population. Conventional adenocarcinoma predominated (97.9%), with one mucinous case. Tumors were located in the rectum (37.5%), left colon (33.3%), and right colon (29.17%). Most tumors were moderately differentiated (Grade II, 91.7%), with T3 invasion in 75%. Lymph node involvement was observed in 50% of patients. Inflammation was mild in 14.6%, moderate in 39.6%, and severe in 45.8%. Desmoplastic reaction was mild in 72.9%, moderate in 22.9%, and severe in 4.17%. Tumor budding was low in 25%, intermediate in 29.17%, and high in 45.83%. Tumor deposits were absent in 87.5%, lymphovascular invasion present in 45.83%, tumor necrosis in 68.75%, and perineural invasion absent in 70.83%. Among patients, 27.08% had an overall survival of less than 3 years, 12.5% survived 3–5 years, and 60.42% survived more than 5 years.

Mutation distribution and co-mutation patterns among CRC patients

Molecular profiling of the CRC cohort revealed distinct mutation patterns, as summarized in Table 1. *NRAS* mutations were the most frequent (52.08%), followed by *KRAS* (47.92%) and *PIK3CA* (16.67%). *KRAS* exon 4 (33.33%) and *NRAS* exon 3 (29.17%) were the most commonly mutated exons, while *KRAS* exon 3 (10.42%) and *PIK3CA* exons 9 (6.25%) and 20 (10.42%) showed lower frequencies. It should be noted that exon-specific mutation frequencies may exceed overall gene-level totals, as some patients harbored concurrent mutations in more than one exon of the same gene, reflecting the multiplex nature of the assay used.

Concurrent mutations in multiple genes were observed in 21 patients, as summarized in Table 2. The most frequent co-mutation involved *NRAS* and *KRAS*, detected in 18 patients (37.50%),

including three patients who also harbored *PIK3CA* mutations. Co-mutations of *KRAS* and *PIK3CA* were identified in 10.42% of cases, while *NRAS* and *PIK3CA* co-mutations were observed in 8.33%. Single-gene mutations were identified in 12 patients, including *NRAS*-only mutations in six patients (12.50%), *KRAS*-only in three patients (6.25%), *PIK3CA*-only in two patients (4.17%), and *BRAF*-only in one patient (2.08%). No isolated *AKT1* mutations were detected.

Correlation between clinicopathological characteristics and gene mutation status in CRC patients

The relationship between clinicopathological features and *NRAS*, *KRAS*, and *PIK3CA* mutations was assessed, as summarized in Supplementary materials, Table 2. No statistically significant associations were observed between gene mutations and age, sex, tumor location, morphology, grade, T stage, lymph node status, or other histopathological parameters. *NRAS* mutations were more frequent in patients <60 years (65% vs. 42.86%), and *PIK3CA* mutations tended to occur more in right-sided tumors (35.71% vs. 12.50% left-sided, 5.56% rectal), though these trends were not significant. Tumor budding, perineural invasion, tumor deposits, and necrosis also showed no correlation with mutation status.

Survival analysis indicated no significant effect of *NRAS* ($p = 0.225$) or *PIK3CA* ($p = 0.451$) mutations. A non-significant trend was observed for *KRAS* mutations, with higher prevalence in patients surviving less than 5 years and lower prevalence in those surviving more than 5 years ($p = 0.070$).

Correlation between clinicopathological characteristics and survival outcomes in CRC patients

The relationship between clinicopathological features and survival is summarized in Table 3. Age and sex did not significantly affect survival. Other parameters, including tumor morphology, location, grade, desmoplastic reaction, lymphovascular invasion, tumor budding, perineural invasion, tumor deposits, necrosis, and T stage, also showed no significant correlations.

Notably, tumor-associated inflammatory response was significantly associated with survival ($p = 0.0276$). Patients with mild inflammation had poorer outcomes, with 57.14% surviving less than 3 years. Moderate inflammation was associated with a favorable prognosis, as 84.2% survived more

than 5 years. Patients with severe inflammation showed intermediate outcomes, with 31.82% surviving less than 3 years, 22.73% surviving 3–5 years, and 45.5% surviving more than 5 years.

Lymph node status was another significant predictor of survival ($p = 0.0351$). Node-negative (N0) patients had the best outcomes, with 79.17% surviving more than 5 years, 16.67% surviving less than 3 years, and 4.17% surviving 3–5 years, whereas N2 patients fared worst, with 44.4% surviving less than 3 years, 33.3% surviving 3–5 years, and 22.2% surviving more than 5 years. These findings underscore the prognostic importance of tumor-associated inflammation and lymph node involvement in colorectal cancer survival.

Longitudinal survival analysis

Kaplan–Meier survival analysis was performed to evaluate patient outcomes over the six-year follow-up period, as presented in Table 4 and Figure 1. Following the first recorded death, the estimated survival probability decreased to 94.12%, with a corresponding mortality rate of 5.88%. Within the first few days (0.01 years), two cumulative deaths had occurred, reducing the survival estimate to 88.24% and increasing the mortality probability to 11.76%, with 16 patients remaining at risk. At three months, the survival function declined to 82.35%, while the mortality probability rose to 17.65%, with 15 patients at risk. At two years, the survival probability decreased to 41.18% and the mortality probability increased to 58.82%; three deaths were recorded at this time point, with 10 patients at risk immediately before these events. By five years, the estimated survival probability was 11.76%, whereas the cumulative mortality probability reached 88.24%. Four patients were at risk immediately before the deaths recorded at five years. At six years, the estimated survival probability declined to 0%, and cumulative mortality reached 100%.

Exploratory multivariable analysis

Because of the limited sample size and the small number of patients in the <3-year survival group, multivariable analysis was performed as an exploratory binary logistic regression using short survival (<3 years) as the outcome. The models were restricted to two predictors to reduce the risk of overfitting. In model 1, *KRAS* mutation and positive lymph node count were entered together. *KRAS* mutation showed a non-significant trend toward higher odds of short survival after adjustment for positive lymph node count. In model 2, *KRAS/NRAS* co-mutation and positive lymph node count were entered together; *KRAS/NRAS* co-mutation also showed a non-significant trend

toward higher odds of short survival. None of the predictors remained statistically significant in the multivariable models. See Table 5.

Discussion

In this study, we analyzed clinicopathological characteristics, molecular alterations, and survival outcomes in 48 CRC patients with follow-up exceeding five years. The relatively low mean age and the substantial proportion of patients under 60 years reflect the increasing incidence of CRC among younger adults, consistent with regional and global trends.^{19,20} The predominance of men (58.3%) aligns with previous studies and may reflect sex-related biological, hormonal, dietary, and lifestyle risk factors.²⁰

The rectum was commonly the most involved site (37.5%), followed by the left and right colon, consistent with regional data from Iraq and neighboring populations.^{21,22} Conventional adenocarcinoma predominated (97.9%), and most tumors were moderately differentiated (91.7%), reflecting global patterns.²³ T3 tumors were most frequent (75%), suggesting delayed presentation, a trend commonly reported in developing healthcare settings.²⁴

Lymph node involvement was present in 50% of patients, with all cases meeting international standards of examining ≥ 12 nodes, supporting accurate staging. Severe tumor-associated inflammatory response was observed in nearly half of patients, highlighting the role of the tumor immune microenvironment in CRC progression.²⁵ High rates of tumor necrosis (68.8%) and tumor budding (45.8%), along with lymphovascular and perineural invasion, indicate aggressive histopathological features associated with poorer prognosis.^{26,27}

Molecular profiling revealed an unexpectedly high prevalence of *NRAS* mutations (52.1%), exceeding the 2–7% typically reported in international cohorts.^{28,29} *KRAS* (47.9%), *PIK3CA* (16.7%), and *BRAF* (2.1%) mutations were within global ranges, while no *AKT1* mutations were detected.³⁰ The unexpectedly high prevalence of *NRAS* mutations and the frequent co-detection of *KRAS* and *NRAS* mutations (37.5%) require critical evaluation. Several contributing factors are proposed. First, the EntroGen multiplex real-time PCR platform has an analytical sensitivity of ≥ 1 –5% mutant allele frequency, substantially exceeding the detection threshold of conventional sequencing methods (15–20%), and may therefore capture subclonal *NRAS* mutations present in minor tumor cell populations that would remain undetected by less sensitive assays.³¹ Second, published multiregion sequencing studies have documented *RAS* Intratumoral Heterogeneity (ITH)

in 33–36% of CRC cases,³² a rate remarkably consistent with our co-detection frequency of 37.5%. When bulk macrodissected FFPE tissue, which captures DNA simultaneously from multiple tumor sub regions, is analyzed by highly sensitive multiplex PCR, co-detection of mutations from spatially distinct subclonal populations is biologically plausible. This interpretation is supported by the aggressive histopathological profile of our cohort, including high tumor budding (45.8%), lymphovascular invasion (45.8%), and tumor necrosis (68.75%), all of which are established markers of clonal diversity. Third, *NRAS* mutation data from the Kurdistan region of Iraq are essentially absent from published literature, Al-Shamsi *et al.* noted that *NRAS* mutations had not been previously characterized in an Arab population,³³ highlighting a gap in regional molecular epidemiological data. Comparison with neighboring cohorts^{34,35} suggests that methodological factors, particularly the high sensitivity of our assay applied to archival FFPE material collected between 2013 and 2015, likely contribute substantially to our elevated detection rates. Fourth, regarding sample admixture, macrodissection of FFPE tissue, while performed under direct microscopic visualization to maximize tumor content, cannot achieve the purity of laser capture microdissection; admixture of adjacent stromal or normal epithelial cells is possible, though this would be expected to dilute rather than generate additional oncogenic mutation signals. Fifth, the possibility of non-specific amplification in degraded archival FFPE DNA contributing to co-detection cannot be entirely excluded in the absence of orthogonal validation. These findings are therefore presented as exploratory observations requiring confirmation in future studies using Next-Generation Sequencing (NGS)-based approaches.

KRAS exon 4 (33.3%) and *NRAS* exon 3 (29.2%) were the most frequently mutated exons, differing from the commonly reported *KRAS* exon 2 predominance, which may indicate regional mutational hotspots or methodological differences.^{36,37}

Clinicopathological correlations showed no significant associations between *NRAS*, *KRAS*, or *PIK3CA* mutations and age, sex, tumor grade, or stage, consistent with prior reports.³⁸ However, trends were observed, including higher *NRAS* mutations in patients under 60 years and in rectal tumors, *KRAS* enrichment in rectal cancers, and increased *PIK3CA* mutations in right-sided tumors, suggesting underlying molecular heterogeneity deserving further study.

Survival analysis revealed a marked decline over six years. Among clinicopathological factors, only tumor-associated inflammation and lymph node status were significantly associated with survival. Moderate inflammatory infiltration correlated with improved outcomes ($p = 0.0276$), emphasizing the prognostic value of host immune response, while node-negative patients exhibited superior

long-term survival ($p = 0.0351$). *KRAS* mutations showed a non-significant trend toward poorer survival, consistent with literature suggesting adverse prognosis and anti-EGFR therapy resistance.^{39,40} The lack of statistical significance may reflect limited statistical power in this small cohort, though a true biological effect cannot be confirmed from the present data.

These findings suggest the potential clinical importance of comprehensive molecular profiling in CRC. The high prevalence of *NRAS* mutations, frequent co-mutations, and atypical exon distribution indicate the potential value of extended RAS testing in this population, pending validation in larger cohorts. Integrating molecular data with established histopathological markers, such as lymph node status and inflammatory response, may improve risk stratification and guide personalized therapy. Importantly, these results originate from an underrepresented region where data on CRC molecular profiles are scarce. To our knowledge, this is the first study in the Duhok region to provide comprehensive analysis including *NRAS* mutations alongside *KRAS* and *PIK3CA* profiling, underscoring the relevance of regional molecular epidemiology for guiding precision oncology.

Several limitations of this study must be acknowledged. First, the sample size ($n=48$) is relatively small, increasing the risk of both false positive and false negative findings, particularly in the context of multiple subgroup and correlation analyses; no correction for multiple comparisons was applied, and all associations should be considered exploratory and hypothesis generating rather than definitive. Second, orthogonal validation of molecular findings, particularly the unexpectedly high *NRAS* mutation frequency using independent methods such as Sanger sequencing or next-generation sequencing was not performed, representing an important constraint on the certainty of our molecular results. Third, FFPE tissue blocks collected between 2013 and 2015 represent archival material in which progressive DNA fragmentation may influence real-time PCR assay performance. Fourth, multivariate survival analysis was limited to two variables due to the small number of events relative to sample size, precluding comprehensive assessment of independent prognostic factors. Fifth, this is a single-center retrospective study from one institution in the Duhok governorate, which may limit the generalizability of findings to the broader Kurdistan region or other populations. Future prospective, multicenter studies with larger cohorts and NGS-based molecular profiling are recommended to validate and expand upon these findings.

Conclusions

CRC patients from the Kurdistan region of Iraq exhibited an unexpectedly high *NRAS* mutation frequency and frequent *KRAS/NRAS* co-mutations relative to international cohorts. These molecular findings are exploratory and require validation in larger independent cohorts using orthogonal methods, particularly next-generation sequencing. Tumor-associated inflammatory response and lymph node status were the only statistically supported clinicopathological predictors of survival in this cohort. These results suggest the potential value of integrated molecular and histopathological evaluation to inform treatment strategies and improve outcomes in CRC patients from underrepresented populations. Multicenter prospective studies with larger sample sizes are recommended to confirm these preliminary findings.

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Table 1. Molecular assay results in CRC patients.

Molecular assays (n=48)	Outcomes no (%)	
	Negative	Positive
<i>NRAS</i>	23 (47.92)	25 (52.08)
<i>KRAS</i>	25 (52.08)	23 (47.92)
<i>PIK3CA</i>	40 (83.33)	8 (16.67)
<i>KRAS</i> exon2	36 (75.00)	12 (25.00)
<i>KRAS</i> exon 3	43 (89.58)	5 (10.42)
<i>KRAS</i> exon 4	32 (66.67)	16 (33.33)
<i>NRAS</i> exon 2	39 (81.25)	9 (18.75)
<i>NRAS</i> exon 3	34 (70.83)	14 (29.17)
<i>NRAS</i> exon 4	35 (72.92)	13 (27.08)
<i>PIK3CA</i> exon 9	45 (93.75)	3 (6.25)
<i>PIK3CA</i> exon 20	43 (89.58)	5 (10.42)

Data are presented as n and %. CRC, colorectal cancer; *NRAS*, neuroblastoma RAS viral oncogene homolog; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *BRAF*, B-Raf proto-oncogene; *AKT1*, AKT serine/threonine kinase 1; PCR, polymerase chain reaction. Mutations detected using the Colorectal Cancer Mutation Detection Panel (EntroGen Inc., USA). Exon subtotals may exceed gene-level totals as some patients harbored concurrent mutations in more than one exon of the same gene

Table 2. Molecular assay outcomes in CRC patients with positive and negative co- mutation and single gene mutation.

Molecular assays (n=48)	Outcomes no (%)	
	Negative	Positive

<i>NRAS</i> with <i>KRAS</i>	30 (62.50)	18 (37.50)
<i>KRAS</i> with <i>PIK3CA</i>	43 (89.58)	5 (10.42)
<i>NRAS</i> with <i>PIK3CA</i>	44 (91.67)	4 (8.33)
<i>NRAS</i> only	42 (87.5)	6 (12.5)
<i>KRAS</i> only	45 (93.75)	3 (6.25)
<i>PIK3CA</i> only	46 (95.83)	2 (4.17)
<i>BRAF</i> only	47 (97.9)	1 (2.08)
<i>AKT1</i> only	48 (100.00)	0 (0.00)

Data are presented as n and %. CRC, colorectal cancer; *NRAS*, neuroblastoma RAS viral oncogene homolog; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *BRAF*, B-Raf proto-oncogene; *AKT1*, AKT serine/threonine kinase 1. Co-mutation defined as concurrent detection of mutations in two or more genes in the same patient sample.

Table 3. Correlation between clinicopathological characteristics and survival rates in CRC patients.

Characteristics (n=48)	Survival rate no (%)			p value
	<3 years	3—<5 years	≥ 5 years	
Age groups				
< 60 years	4 (20.00)	4 (20.00)	12 (60.00)	0.3364
≥ 60 years	9 (32.14)	2 (7.14)	17 (60.71)	
Gender				
Men	9 (32.14)	3 (10.71)	16 (57.14)	0.6295
Women	4 (20.00)	3 (15.00)	13 (65.00)	
Macroscopic appearance				
Annular mass	4 (36.36)	2 (18.18)	5 (45.45)	0.5629
Fungating mass	5 (27.78)	3 (16.67)	10 (55.56)	
Ulcerating mass	4 (21.05)	1 (5.26)	14 (73.68)	
Tumor location				0.4132

Left side	5 (31.25)	0 (0.00)	11 (68.75)	
Right side	4 (28.57)	2 (14.29)	8 (57.14)	
Rectum	4 (22.22)	4 (22.22)	10 (55.56)	
Inflammatory reaction				
Mild	4 (57.14)	0 (0.00)	3 (42.86)	0.0276
Moderate	2 (10.53)	1 (5.26)	16 (84.21)	
Severe	7 (31.82)	5 (22.73)	10 (45.45)	
Desmoplastic reaction				
Mild	8 (22.86)	5 (14.29)	22 (62.86)	0.8190
Moderate	4 (36.36)	1 (9.09)	6 (54.55)	
Severe	1 (50.00)	0 (0.00)	1 (50.00)	
Tumour grade				
Poor	1 (50.00)	0 (0.00)	1 (50.00)	0.7279
Moderate	12 (27.27)	6 (13.64)	26 (59.09)	
Well	0 (0.00)	0 (0.00)	2 (100)	
Depth of invasion				
T1	0 (0.00)	0 (0.00)	1 (100.00)	0.1408
T2	4 (40.00)	0 (0.00)	6 (60.00)	
T3	9 (25.00)	5 (13.89)	22 (61.11)	
T4	0 (0.00)	1 (100.00)	0 (0.00)	
Positive lymph node				
N0	4 (16.67)	1 (4.17)	19 (79.17)	0.0351
N1	5 (33.33)	2 (13.33)	8 (53.33)	
N2	4 (44.44)	3 (33.33)	2 (22.22)	
Lymphovascular invasion				
Negative	7 (26.92)	2 (7.69)	17 (65.38)	0.5270
Positive	6 (27.27)	4 (18.18)	12 (54.55)	
Tumour budding				
Low (0-4) buds	4 (33.33)	0 (0.00)	8 (66.67)	0.5380
Intermediate (5-9) buds	4 (28.57)	3 (21.43)	7 (50.00)	
High (10 or more) buds	5 (22.73)	3 (13.64)	14 (63.64)	
Perineural invasion				
Not present	8 (23.53)	3 (8.82)	23 (67.65)	0.2450
Present	5 (35.71)	3 (21.43)	6 (42.86)	

Tumour deposits				
Not present	11 (26.19)	4 (9.52)	27 (64.29)	0.1909
Present	2 (33.33)	2 (33.33)	2 (33.33)	
Tumour necrosis				
Not present	3 (20.00)	2 (13.33)	10 (66.67)	0.7568
Present	10 (30.30)	4 (12.12)	19 (57.58)	

Data are presented as n and %. CRC, colorectal cancer; LVI, lymphovascular invasion; and PNI, perineural invasion.

Table 4. Longitudinal analysis of survival and mortality during follow-up among the subgroup of colorectal cancer patients included in the Kaplan–Meier analysis (n=17).

Follow-up period (years)	Survival probability%	Mortality probability%	Standard error of survival	Number of deaths	Number censored	Number at risk
0.00	100.00	0.00	0.0000	0	0	17
0.00	94.12	5.88	0.0571	1	0	17
0.01	88.24	11.76	0.0781	1	0	16
0.30	82.35	17.65	0.0925	1	0	15
0.50	76.47	23.53	0.1029	1	0	14
1.00	58.82	41.18	0.1194	3	0	13
2.00	41.18	58.82	0.1194	3	0	10
3.00	35.29	64.71	0.1159	1	0	7
4.00	23.53	76.47	0.1029	2	0	6
5.00	11.76	88.24	0.0781	2	0	4
6.00	0.00	100.00	0.0000	2	0	2

Note: survival and mortality probabilities were estimated using Kaplan–Meier analysis. Mortality probability was calculated as one minus the survival probability. CRC, colorectal cancer.

Table 5. Logistic regression analysis for short survival (<3 years).

Model	Predictor	Odds ratio	95% CI	p value
Univariable	<i>KRAS</i>	3.38	0.87- 13.13	0.079
Univariable	<i>KRAS/NRAS</i> co-mutation	2.55	0.69-9.37	0.160
Univariable	Positive lymph nodes	1.13	0.90-1.41	0.292

Univariable	Severe inflammatory reaction	1.56	0.43-5.59	0.499
Univariable	Stage III-IV	1.35	0.37-4.95	0.653
Multivariable model 1	<i>KRAS</i>	3.31	0.84- 13.04	0.088
Multivariable model 1	Positive lymph nodes	1.12	0.89-1.41	0.334
Multivariable model 2	<i>KRAS/NRAS</i> co-mutation	2.42	0.65-9.04	0.190
Multivariable model 2	Positive lymph nodes	1.11	0.89-1.39	0.357

Note. Outcome: survival duration <3 years versus ≥ 3 years. Models are exploratory because the sample size was small. Odds ratios for positive lymph nodes are per one additional positive lymph node. CI, confidence interval.

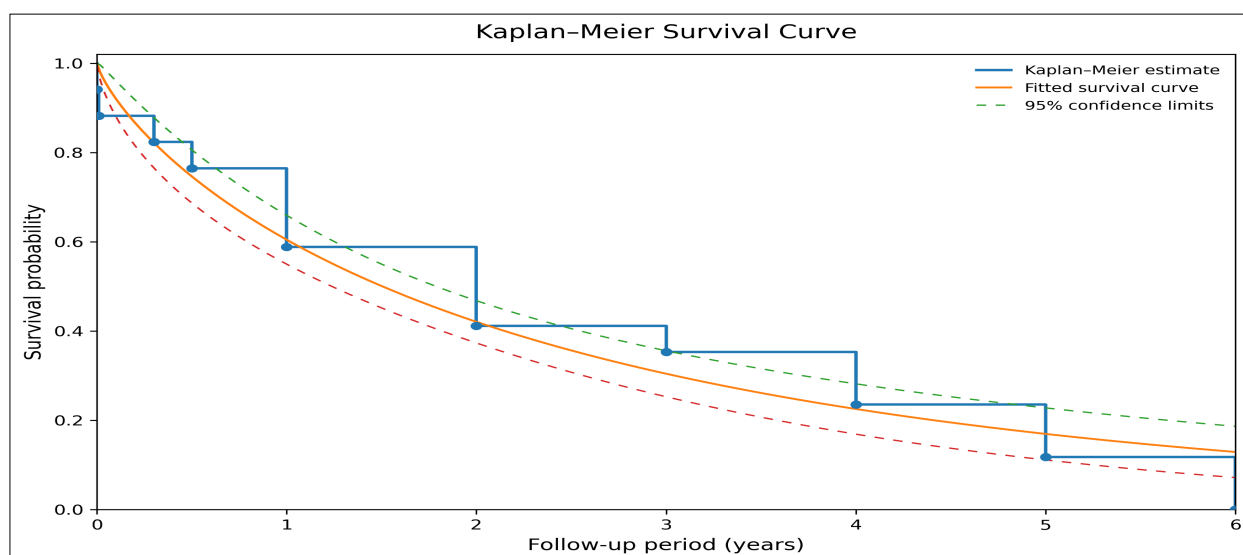


Figure 1. Kaplan-Meier survival curve during the six-year follow-up period among the analyzed subgroup of colorectal cancer patients (n=17).

Note. The step line represents the Kaplan-Meier survival estimate, the middle smooth line represents the fitted survival curve, and the dotted lines represent its approximate 95% confidence limits. No censored observations were recorded in this subgroup.

Contributions: Jiyan Ali Haji conceptualized and designed the study, supervised the molecular analyses, performed data interpretation, and prepared the original draft, being primarily responsible for manuscript writing. Dijwar Ali Haji was involved in the molecular practical work and, together with Mayda Ilias Yalda, performed the pathological examinations and contributed to clinicopathological correlation. Ghazwan Fawzi Ahmed participated in data interpretation and contributed to manuscript writing. All authors reviewed, revised, and approved the final version of the manuscript.

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Online supplementary materials

Table1. Clinical and histopathological characteristics of patients.

Table 2. Correlation of clinicopathological characteristics and survival rate with positive and negative gene mutations in *NRAS*, *KRAS*, and *PIK3CAP* among CRC patients.