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Impact of *RAS/RAF* mutations on clinical and prognostic outcomes in metastatic colorectal cancer

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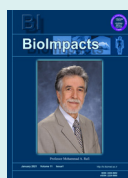[2] Tabriz Health Services Management Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
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Article Info

Abstract

Introduction: Early-activated *RAS/RAF* mutation status is a key molecular finding in colorectal cancer (CRC), while these mutations have been proposed as predictive and prognostic biomarkers. The present study has been designed as a longitudinal study to evaluate and summarize the different genotypes of metastatic CRC (mCRC), and assessing any association with the disease prognosis and clinicopathological characteristics. This study was performed in two main referral hospitals of Tabriz University of Medical Sciences, over three years (2016-2018).

Methods: Mutations were detected by Idylla tests of *KRAS/NRAS/BRAF* among a total of 173 mCRCs, using surgically-resected specimens or biopsied samples. To evaluate the factors associated with overall survival (OS) and prognosis, the Cox proportional hazards model was used in two steps to estimate the outcome measures (hazard ratio, or HR) with a 95% confidence interval (CI).

Results: The nominal 1 to 5-year OS rates were 78%, 65%, 55%, 46%, and 42%, respectively. *KRAS* mutations in codon 12 was an independent significant prognostic factor, as the patients with codon 12 mutations had a significantly lower OS (P Log-rank = 0.049) and a higher hazard of mortality (HR = 2.30; 95% CI: 0.95-5.58; *P* = 0.066). Also, the mCRC patients with liver metastasis (HR = 2.49; 95% CI: 1.49-12.52; *P* = 0.002) and tumors of the distal colon (HR = 3.36; 95% CI: 1.07-10.49; *P* = 0.037) had a significantly worse prognosis.

Conclusion: *KRAS* mutation in codon 12 was an independent significant poor prognostic factor, and patients with liver metastasis had a significantly worse prognosis. Routinely performing specific oncogenic tests may help improve the patients' prognosis and life expectancy.
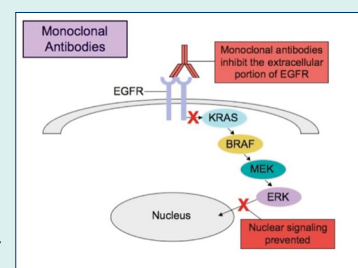
Introduction

Colorectal cancer (CRC) is one of the main leading causes of cancer-related death worldwide that ranks as the second fatal cancer and accounted for around one in ten cancer deaths in 2018.[1] CRC ranked as the third most common cancer in Iran, which accounts for 9% of all cancers in the country. However, it is second most common cancer in East Azerbaijan.[2] Despite the long-standing screening and early detection programs as well as improvements in cancer treatment and management using targeted therapies in most developed countries, its incidence and mortality have shown an increasing trend in some regions of the world, especially Asia and developing countries.[1,3,4] CRC represents a heterogeneous genetic and epigenetic alteration that could lead to different phenotypes. Chromosomal instability pathway, including the mutational activation of specific proto-oncogenes, is the most common type of mutagenic pathway in CRC.[5,6] Early-activated *RAS/RAF* mutation status is a key molecular finding, especially in metastatic CRC (mCRC), and tests are therefore recommended in stage IV of CRC at the time of diagnosis for targeted therapy approaches.[7]

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These mutations have been proposed as predictive and prognostic biomarkers in mCRC.[8] Besides, KRAS gene mutation, with a 90% occurrence rate, is localized in codons 12 and 13. Herein, this point mutation can be used as a predictive biomarker, to improve the efficacy of anti-*EGFR* targeted therapies.[9,10] Research has also suggested that the pattern of specific *KRAS* gene mutation, and so the impact of specific amino acid changes, are associated with different prognostic values and responses to anti-*EGFR* therapies.[9,11] Although some researchers have suggested that the presence of specific mutations in CRC is associated with the metastasis and outcome potential,[4,9] but there is still an inconsistency in the available data about.[4,12,13]

As very few studies have analyzed the pattern of different point mutations in Iranian mCRC, the present study was conducted to evaluate and summarize the different genotypes of mCRC and its association with the disease prognosis and OS as well as clinicopathological characteristics. This research also seeks to find any possible associations between *KRAS*, *NRAS*, and *BRAF* mutations in mCRC and primary tumor location and metastatic site.

Materials and Methods
Study design
This longitudinal study is performed in three phases:

Cross-sectional phase: Based on our inclusion criteria, we collected data for 264 mCRCs, with histo-pathologically-confirmed CRC and imaging- and/or surgery-confirmed metastasis. Standard demographic characteristics, clinicopathological data, and mutation subtypes at the time of diagnosis were recorded for each patient using the hospital database or through the patients' follow-up. These variables included data on somatic mutations (*KRAS*, *NRAS*, and *BRAF*) and type of mutation, age at diagnosis, gender, primary tumor anatomic site (proximal and distal colon and rectum), metastatic site (lymph node, brain, lung, bone, omentum, liver, etc.), morphologic type (adenocarcinoma, etc.), and tumor grade (well, moderately, and poorly differentiated). The ethics committee of Tabriz University of Medical Sciences has been approved this project (IR.TBZMED.REC.1395.18), and all patient's information and records are confidential.

Follow-up phase: For OS analysis, we contacted the patients or their family, by following exact ethical rules for following, and getting their consent.

Analytical case only phase: The relationship between clinicopathological variables and evaluated mutations has been assessed in mCRC patients. Structured questionnaires were used to collect more information about the main risk factors of the disease, including smoking, alcohol intake, marital status, body mass index, positive family history of CRC, occupation, education, and immobility. These questionnaires had been formally validated in a previous study by the same researchers.[14]

Alcohol intake was evaluated in terms of frequency,

quantity, and type of alcohol consumed (beer, wine and/or spirits), and the level of intake was rated as 'never', 'low' (less than once a week, one glass), moderate (two-three times a week, one to two glasses) and 'high' (more than three times per week, more than two glass). To achieve better regression analysis results, the patients were divided into two subgroups, including 'no drinking' (never or low consumption) and 'drinkers' (moderate or high consumption) groups. As for the smoking status, the patients were asked about their number of cigarettes smoked per day (less or more than one box) and the duration of smoking (over the span of one year). To achieve better regression analysis results, the patients were divided into subgroups, including 'no smoking' (never smoked during the last 20 years) and 'smokers' (smoking at least one cigarette per day, at least for one year over the last 20 years). As for the other lifestyle factors, the responses were designed based on a Likert scale. The occupation was categorized as 'employed' (full- or part-time) and 'retired or unemployed' (student or without a job). The subjects' education was categorized as 'illiterate', 'primary/high school education', and 'university education'. As for the place of residence, the patients were asked if they were owners or otherwise (including renting, non-fixed status, homeless). The subjects were categorized under three subgroups in terms of their body mass index (BMI), including the 'BMI≤25' group, the '25-30 BMI' group, and the 'higher than 30 BMI' group. Also, age was categorized to <50 and ≥50, and age 50 years was taken as a reasonable cutoff point.

Study setting
This research was performed in two main referral hospitals of Tabriz University of Medical Sciences, Imam Reza, and Shahid Ghazi educational and treatment centers, using the method of "gathering all eligible samples" (having our inclusion criteria) over three years, during April 2016 to April 2018. All molecular tests were performed by the co-investigate pathologist of this study, at the reference molecular laboratory, with available standard molecular tests.

Mutation status
The patients underwent *RAS*/*RAF* type specification using surgically-resected specimens or biopsied samples of their primary tumors, and molecular testing was routinely prescribed by their medical oncologists.

After confirming their mCRC, an expert pathologist undertook the histological examination of their cancer tissues. Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections (5–30 μm) from the samples underwent molecular testing for *KRAS*, *NRAS*, and *BRAF* mutations. Mutations were detected by Idylla tests of *KRAS/NRAS/BRAF* (Biocartis NV, 2800 Mechelen, Belgium, BCT006631). According to the reference clinical guidelines,[15] the *KRAS* gene mutations detected for codons 12, 13, 59, 61, 117 and

146 at the study's reference molecular laboratory included Gln61His (c.183G>C), Gly12Ala (c.35G>C), Gly12Asp (c.35G>A), Gly12Cys (c.34G>T), Gly12Cys (c.34G>T), Gly12Ser (c.34G>A), Gly12Val (c.35G>T), Gly13Asp (c.38G>A) and Gly13Asp. The *NRAS* mutation detected was Q61R (c.182A>G) and the *BRAF* mutation detected V600E/D (c.1799T>A).

The Turnaround Time (TAT) from sample shipping to getting the mutation results were under ten days for more than 95% of the cases. Three to four FFPE tissue sections (5-30 μm) of CRC tumors were loaded in cartridges and then inserted into the system according to the manufacturer's instructions. After the DNA extraction and purification by the system, multiplex-PCRs were performed, and finally, *KRAS/NRAS/BRAF*-specific software was used to automatically determine the presence of the noted mutations.

Statistical analysis

Given that only one *NRAS* mutation and one *BRAF* mutation were observed, all the statistical analyses were performed on the cases with *KRAS* gene mutations. All the statistical analysis was performed using STATA (MP 14.2, Stata Corp LP, College Station, Texas 77845 USA). Pearson's Chi-Square test was used for the subgroup analysis and to test the differences in each of the studied variables in connection to the *KRAS* gene mutation subtypes. All the clinicopathological variables were stratified as two common types of *KRAS* mutations (codons 12 and 13). For the CRC-specific survival analysis, the overall survival (OS) was defined as the length of time between the pathological or surgical diagnosis until death (CRC-specific) or last follow-up time. The patients who were alive at the time of the last follow-up were considered censored in the survival analysis. The overall, one-, three- and five-year survival were estimated using the timetable survival method. The prognosis was evaluated as the OS, and subgroup analysis was performed to compare the survival function in the different mutation subtypes using Kaplan-Meier survival estimate with log rank (Mantel-Cox) test. All the other non-specific CRC mortality data were excluded.

To evaluate the factors associated with OS and prognosis, the Cox proportional hazards model was used in two steps (univariate and multivariate steps) to estimate the hazard ratio (HR) with a 95% Confidence Interval (CI). Also, the proportional hazards assumption has been tested, based on Schoenfeld residuals (ph-test). First, the unadjusted HRs were estimated for each variable and the tumor characteristics. Then, the multiple regression modeling was used based on the forward stepwise model to calculate the adjusted HRs in the presence of confounding variables (age, gender, grade, smoking status, alcohol intake, tumor subsite, metastatic site, and morphologic type). At the final step, the Poisson regression model was applied to confirm the adjusted HR results in the presence of competing risks.

All the tests were two-tailed and *P*<0.05 was set as the level of statistical significance.

Results

Frequency of KRAS mutation types and their association with different clinicopathological characteristics

We enrolled 264 mCRC cases during three years of study. From these, we excluded 58 cases because of missing some main data, five CRCs had nonspecific deaths (other than CRC), and we lost to follow up for 28 CRC cases (because of changed contact information, or moving of patients). At the final step, a total of 173 patients were enrolled in this analytical research study (Fig. 1). Of the 106 cases with *KRAS* mutation data, 45 (42.5%) were positive for *KRAS* gene mutation and 61 cases (57.5%) had the wild-type gene. Mutations in exon 2 were identified in 44 of the cases, including 32 (71.1%) with point mutations in codon 12 and 12 (26.7%) with a single mutation in codon 13, and only one case (2.2%) had a point mutation in exon 61 of the *KRAS* gene. *NRAS* mutations were tested in 75 mCRC samples, and only one case was positive for *NRAS* mutation (Q61R (c.182A>G)), and of the 39 cases tested for *BRAF* mutation, only one case had a mutation in *BRAF* codon 600 (V600E). Because of missing data of *NRAS* and *BRAF* mutations in most of the cases, they were not included in the statistical analysis.

From the 173 enrolled mCRC samples, 97 cases (56.1%) were male and 76 cases (43.9%) were female. Their mean age was 58.91 ± 12.95 years (range: 21 to 90 years). Most patients were ≥50 years old (n=130, 75.1%) and the most common metastatic site was the liver (n=46, 26.6%), followed by the omentum (n=23, 13.3%). The most common subsite of the primary tumor was the rectum (n=99, 57.2%), with the majority of the tumors being well-differentiated adenocarcinoma (n=67, 38.7%). *KRAS* codon 13 mutations were more common in the male participants and the distal colon, while *KRAS* codon 12 was most obvious in the rectum lesions and the colorectal patients with liver metastasis. The tumors that were well-differentiated and were adenocarcinoma in terms of morphologic type had more *KRAS* 12 mutations. There were no significant associations between these clinicopathological characteristics and the *KRAS* mutation type. The presence of *KRAS* 12, 13, and 61 mutations was not significantly associated with any specific risk factors (smoking, alcohol intake, education, occupation, marital status, immobility, and BMI). Table 1 presents the associations between *KRAS* mutation and different demographic, clinicopathological, and lifestyle factors.

Survival analysis

The median follow-up time was 17 months (0.33 to 71.97 months) and 65 CRC patients (37.6%) died during the follow-up. The mean and median OS were 39.44 (95% CI: 34.86-44.02) and 42.27 (95% CI: 35.56-48.97), respectively. The nominal 1-year, 2-year, 3-year, 4-year, and 5-year
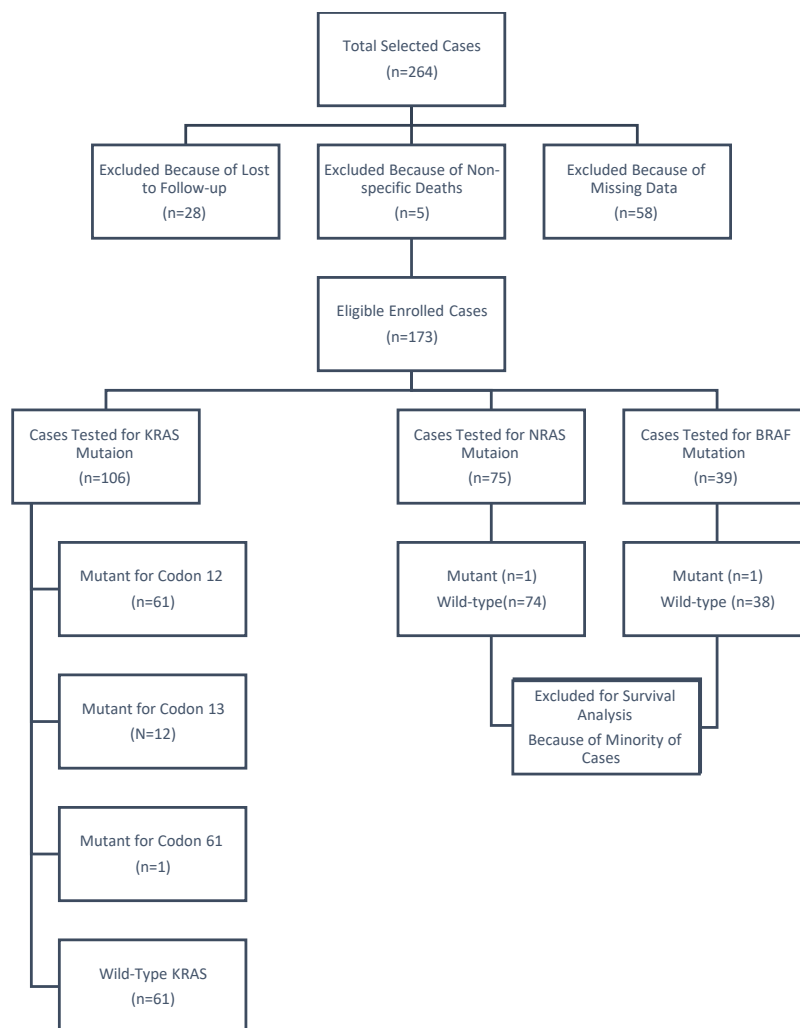
Fig. 1. Flow diagram of study, including and excluding cases.

survival were 78%, 65%, 55%, 46%, and 42%, respectively.

The median OS was 42.27 (95% CI: 27.93-56.60) months for the colorectal patients with wild-type *KRAS* gene. Kaplan-Meier survival estimate didn't show any significant difference in OS between *KRAS* mutant and the wild-type gene (P Log-rank = 0.214) (Fig. 2A). The median OS for the patients with mutations in codon 12 of the *KRAS* gene was 36.17 (95% CI: 18.18-54.15) and the log rank (Mantel-Cox) test for survival distribution of the different *KRAS* mutations showed that *KRAS* codon 12 point mutation was independently associated with a worse OS compared to the wild-type tumors (Log-rank P=0.049) (Fig. 2B). For the patients with mutations in codon 13 of the *KRAS* gene, the median OS was 46.35 (95% CI: 30.97-61.74) and was not associated with a worse OS compared to the wild-type tumors (Log-rank P=0.575). Kaplan-Meier survival estimate did not show any significant difference in OS between *KRAS* codon 12 and 13 mutations and wild-type genes (P Log-rank=0.102) (Fig. 2C). Nonetheless, the OS was 34.4%, 66.7%, and 69.4% in the *KRAS* codon 12 and 13 mutations and the wild-type *KRAS* mutations,

respectively, and there was only one case of a *KRAS* 16 mutation, which remained alive.

Cox regression hazard function analysis for different clinicopathological characteristics

The Cox regression analysis showed that mCRC patients with mutations in codon 12 of the *KRAS* gene had a worse OS (HR=1.77; 95% CI: 0.95-3.30) and the female patients had a worse prognosis than the males (HR=1.3; 95% CI: 0.69-1.85). Smoking in any amount and duration increased the mortality hazard by about 1.5 times (HR=1.46; 95% CI: 0.82-2.57), but alcohol consumption did not have a significant association with the patients' prognosis. The metastatic patients with liver metastasis had a worse OS (HR=1.59; 95% CI: 0.72-3.50) and the patients with rectal tumors had a higher hazard of mortality compared to those with tumors in the other subsites (HR=1.45; 95% CI: 0.69-3.03). Multivariate Cox regression model after adjusting the included risk factors, showed that patients with codon 12 mutations had a lower OS (HR=2.30; 95% CI: 0.95-5.58; *P*=0.066). In comparison, *KRAS* mutations

Table 1. Baseline association between wild-type and mutant *KRAS* subtypes and clinicopathological aspects in mCRC
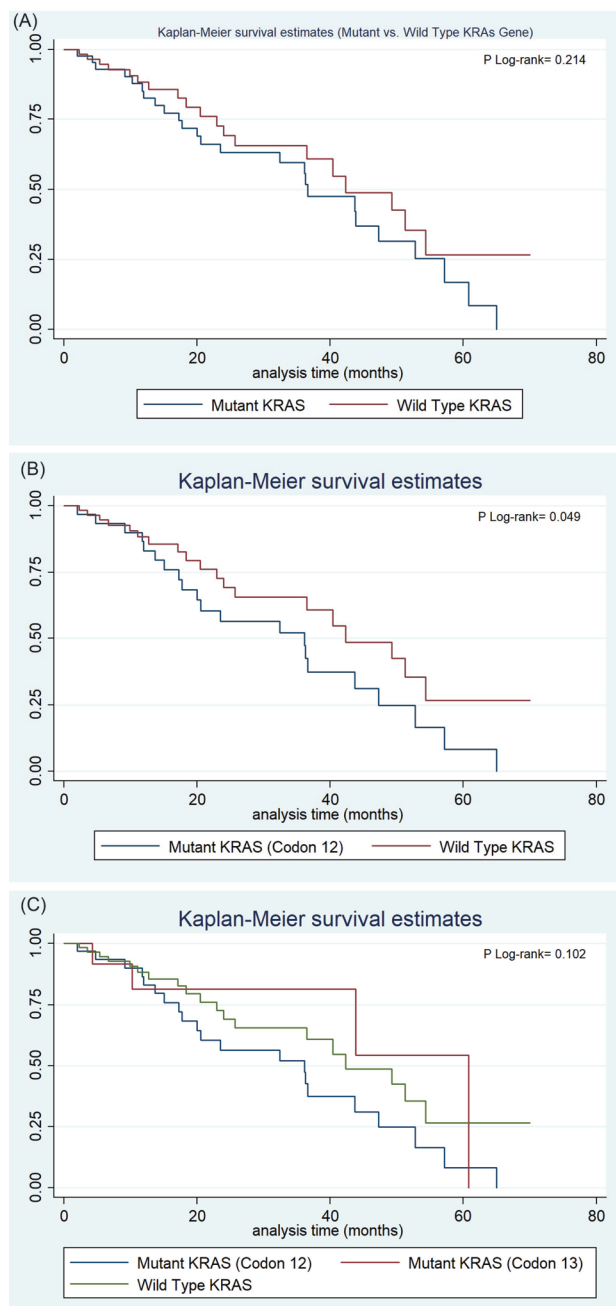
Variable		*KRAS* WT N=62 (57.9%)	*KRAS* Mutant, N=45 (43.9)		P value
			Codon 12 N=32 (72.7%)	Codon 13,16 N=13 (28.2%)	
		No. (%)	No. (%)	No. (%)	
Sex	Female (n=45)	27 (25.2)	16 (15.0)	3 (2.8)	0.147
	Male (n=61)	35 (32.7)	1615.0)	10 (9.3)	
Age	<50 (n=32)	12 (11.2)	15 (14.0)	4 (3.7)	0.018
	≥50 (n=75)	50 (46.7)	17 (15.9)	8 (7.5)	
Morphological type	Adenocarcinoma (n=94)	53 (49.5)	29 (27.1)	12 (11.2)	0.836
	Others (n=13)	9 (8.4)	3 (2.8)	1 (0.9)	
Grade (differentiated)	Well (n=45)	22 (20.6)	17 (15.9)	6 (5.6)	0.482
	Moderate (n=37)	25 (23.4)	7 (6.5)	5 (4.7)	
	Poor (n=25)	15 (14.0)	8 (7.5)	2 (1.9)	
Metastatic Site	Lymph node (n=10)	4 (3.7)	5 (4.7)	1 (0.9)	0.212
	Brain (n=12)	5 (4.7)	6 (5.6)	1 (0.9)	
	Lung (n=11)	10 (9.3)	1 (0.9)	0 (0.0)	
	Bone (n=17)	10 (9.3)	6 (5.6)	1 (0.9)	
	Omentum (n=18)	12 (11.2)	2 (1.9)	4 (3.7)	
	Liver (n=26)	13 (12.1)	9 (8.4)	4 (3.7)	
	Others (n=13)	8 (7.5)	3 (2.8)	2 (1.8)	
Subsite	Proximal (n=20)	13 (12.1)	6 (5.6)	1 (0.9)	0.118
	Distal (n=33)	13 (12.1)	12 (11.2)	8 (7.4)	
	Rectum (n=54)	36 (33.6)	14 (13.1)	4 (3.7)	
Marital Status	Married (n=98)	57 (53.3)	29 (27.1)	12 (11.2)	0.987
	Unmarried (n=9)	5 (4.7)	3 (2.8)	1 (0.9)	
Positive Family History of CRC	Yes (n=21)	13 (12.4)	6 (5.7)	2 (1.8)	0.165
	No (n=84)	48 (45.7)	25 (23.8)	11 (10.5)	
Occupation	Employed (n=25)	9 (8.4)	10 (9.3)	6 (5.6)	0.082
	Retired (n=31)	22 (20.6)	6 (5.6)	3 (2.8)	
	Unemployed (n=51)	31 (29.0)	16 (15.0)	4 (3.7)	
Education	University (n=16)	9 (8.5)	3 (2.8)	4 (3.8)	0.155
	School (n=68)	39 (36.8)	22 (20.8)	5 (6.6)	
	Illiterate (n=22)	14 (13.20	6 (5.7)	2 (1.9)	
Residence	Private (n=86)	52 (48.6)	22 (20.6)	11 (10.3)	0.218
	Rental/Unstable (n=21)	10 (9.3)	10 (9.3)	1 (0.9)	
Immobility	Yes (n=46)	26 (24.3)	14 (13.1)	6 (5.6)	0.712
	No (n=61)	36 (33.6)	18 (16.8)	7 (6.5)	
Smoking	Yes (n=21)	10 (9.3)	7 (6.5)	5 (4.6)	0.339
	No (n=86)	52 (48.6)	25 (23.4)	8 (7.5)	
Drinking	Yes (n=15)	7 (6.5)	5 (4.7)	3 (2.8)	0.375
	No (n=92)	55 (51.4)	27 (25.2)	10 (9.3)	
BMI	≤25 (n=57)	31 (29.0)	19 (17.8)	7 (6.5)	0.369
	25-30 (n=37)	24 (22.4)	9 (8.4)	4 (3.7)	
	≥30 (n=13)	7 (6.5)	4 (3.7)	2 (1.9)	

CRC: colorectal cancer; BMI: body mass index.

in codon 13 had no significant impact on the patients' prognosis (HR=0.52; 95% CI: 0.11-2.55; *P*=0.420). Smoking increased the hazard of mortality by about 7 times (HR=7.32; 95% CI: 2.29-23.28; *P*=001). Also, the mCRC patients with liver metastasis (HR=2.49; 95% CI: 1.49-12.52; *P*=0.002) and tumors of the distal colon (HR=3.36; 95% CI: 1.07-10.49; *P* = 0.037) had a significantly worse OS (Table 2). The Poisson regression analysis showed exactly the same results for Cox regression multivariate analysis.

Also, the proportional hazards assumption test showed that all the variables contributed to each model satisfied the PH assumption of the Cox regressions globally (chi2 = 29.83, *P* = 0.231).

Estimated power for Cox PH regression Wald test, for log-hazard metric by alpha = 0.050 (two-sided), and 0.05 significance, the power of this survival analysis was 0.950 with a sample size of 173 cases.

(A) Kaplan-Meier survival estimates (Mutant vs. Wild Type KRAs Gene)
P Log-rank= 0.214

(B) Kaplan-Meier survival estimates
P Log-rank= 0.049

(C) Kaplan-Meier survival estimates
P Log-rank= 0.102

Fig. 2. (A) Survival Estimated Difference between *KRAS* mutant and the wild-type gene. (B) Survival Estimated Difference between *KRAS* codon 12 mutation independently and wild-type gene. (C) Survival Estimated Difference between *KRAS* codon 12 and 13 mutations and wild-type gene.

Discussion

The impact of specific oncogenic mutations concerning different clinicopathological characteristics was assessed on the prognosis and OS of patients with metastatic CRC. This analytical research study enrolled a total of 173 mCRC patients, and 65 CRC patients (37.6%) died during the follow-up time and the nominal 1-year through 5-year survival was 78%, 65%, 55%, 46%, and 42% respectively. The OS was 34.4%, 66.7%, and 69.4%

in the *KRAS* codon 12 and 13 mutations and the wild-type *KRAS* mutations, respectively, and the *KRAS* codon 12 point mutation was independently associated with a worse OS compared to the wild-type tumors (Log-rank P=0.049). The Cox-Regression Hazard Model also showed that the mCRC patients with codon 12 mutations had a nearly 2 times higher hazard than the codon 13 and *KRAS* wild-type mutations. Smoking strongly increased the hazard of mortality by about 7 times and the mCRC patients with liver metastasis and tumors of the distal colon had a significantly worse OS with HRs of 2.49 and 3.36, respectively. *KRAS* mutations were positive in 42.5% of the cases and only one positive case was found for the *NRAS* mutations (1.3%), and of the 39 cases tested for *BRAF* mutations, only one case (2.6%) had mutations in *BRAF* codon 600 (V600E). These results are similar to other reports from Iran and recent surveys.[4,9] Despite the many studies on the clinical relevance of single point mutations in CRC, this study sought to present a more comprehensive and analytical survey of the prognostic impact of *KRAS* mutation subtypes in mCRC in the northwest of Iran as part of a larger analytical study in this region and had a median follow-up time of 17 months (0.33 to 71.97 months).

Despite the well-recognized role of *KRAS* mutations in the poor efficacy of targeted therapies (anti-*EGFR*) in metastatic CRCs, their oncogenic and prognostic effects remain controversial.[6,16,17] Several studies have shown that *KRAS* mutations are significantly associated with poor prognosis and OS[6]; however, some other studies have demonstrated that OS and disease-free survival do not differ significantly, not only for mutant genes compared to the wild-type but also for different *KRAS* mutation subtypes.[16] Although there is some evidence on the alterations of specific *KRAS* mutations with tumor phenotypes,[9,18,19] limited studies have shown any differences between the patients' prognosis in terms of different *KRAS* mutation types.[20] The present study showed that patients with *KRAS* codon 12 mutations have a significantly worse OS compared to those with the wild-type, but this difference was not significant for *KRAS* codon 13 mutations. The present findings were similar to the results of some previous studies,[4] and inconsistent with the results of some others. Summer et al reported that both codon 12 and 13 mutations had a worse OS, but the hazard function of *KRAS* codon 13 mutations was higher than that of codon 12 mutations (HR=1.53 vs. 1.44) while *NRAS* mutations showed no differences in the prognosis of CRCs and *BRAF* mutations were strongly associated with a worse OS.[21] A recent systematic review and meta-analysis showed that although *KRAS* codon 13 mutations have a worse OS compared to patients with the wild-type gene (pooled HR=1.76), there were no significant differences between *KRAS* codon 12 and 13 mutations in terms of OS in CRC patients.[11]

Table 2. Results of Cox regression analysis of clinicopathologic factors with KRAS mutation types

Variable		Univariable analysis		Multivariable analysis	
		HR† (95% CI)	P value	HR* (95% CI)	P value
KRAS mutation	Codon 12 (n=32)	1.77 (0.95-3.29)	0.073	2.30 (0.95-5.58)	0.066
	Codon 13 (n=12)	0.76 (0.26-2.24)	0.616	0.52 (0.11-2.55)	0.420
	Wild-type (n=62)	1 (Reference)	-	1 (Reference)	-
Sex	Female (n=45)	1.13 (0.69-1.85)	0.634	1.21 (0.45-3.29)	0.710
	Male (n=61)	1 (Reference)	-	1 (Reference)	-
Age	<50 (n=32)	1 (Reference)	-	1 (Reference)	-
	≥50 (n=75)	1.18 (0.66-2.11)	0.584	2.59 (0.92-7.28)	0.071
Morphological type	Adenocarcinoma (n=94)	1.15 (0.58-2.29)	0.683	1.46 (0.38-5.56)	0.580
	Others (n=13)	1 (Reference)	-	1 (Reference)	-
Grade (differentiated)	Well (n=45)	1.02 (0.56-1.86)	0.949	0.34 (0.11-1.10)	0.071
	Moderate (n=37)	0.75 (0.40-1.39)	0.359	0.35 (0.10-1.18)	0.090
	Poor (n=25)	1 (Reference)	-	1 (Reference)	-
Metastatic site	Lymph Node (n=10)	1.07 (0.37-3.16)	0.897	0.24 (0.05-1.22)	0.086
	Brain (n=12)	0.76 (0.30-1.95)	0.570	1.20 (0.20-7.40)	0.844
	Lung (n=11)	1.09 (0.45-2.63)	0.851	0.73 (0.16-3.41)	0.686
	Bone (n=17)	1.10 (0.43-2.87)	0.839	0.43 (0.74-2.43)	0.336
	Omentum (n=18)	0.69 (0.22-1.89)	0.417	1.09 (0.25-4.84)	0.909
	Liver (n=26)	1.59 (0.72-3.50)	0.248	2.49 (1.49-12.52)	**0.002**
	Others (n=13)	1 (Reference)	-	1 (Reference)	-
Subsite	Proximal (n=20)	1 (Reference)	-	1 (Reference)	-
	Distal (n=33)	1.14 (0.51-2.52)	0.750	3.36 (1.07-10.49)	**0.037**
	Rectum (n=54)	1.45 (0.69-3.03)	0.324	2.52 (0.90-7.01)	0.077
Marital status	Married (n=98)	1.32 (0.56-3.08)	0.524	1.52 (0.46-5.05)	0.495
	Unmarried (n=9)	1 (Reference)	-	1 (Reference)	-
Positive family History of CRC	Yes (n=21)	1 (Reference)	-	1 (Reference)	-
	No (n=84)	1.40 (0.69-2.83)	0.355	0.85 (0.25-2.85)	0.792
Occupation	Employed (n=25)	1 (Reference)	-	1 (Reference)	-
	Retired (n=31)	1.27 (0.61-2.63)	0.528	N/A	N/A
	Unemployed (n=51)	1.24 (0.69-2.23)	0.482	N/A	N/A
Education	University (n=16)	1.27 (0.52-3.11)	0.597	N/A	N/A
	School (n=68)	1.35 (0.76-2.41)	0.313	N/A	N/A
	Illiterate (n=22)	1 (Reference)	-	1 (Reference)	-
Residence	Private (n=86)	1.04 (0.54-1.99)	0.913	0.98 (0.26-3.78)	0.981
	Rental/Unstable (n=21)	1 (Reference)	-	1 (Reference)	-
Immobility	Yes (n=46)	1 (Reference)	-	1 (Reference)	-
	No (n=61)	1.22 (0.73-2.04)	0.439	N/A	N/A
Smoking	Yes (n=21)	1.46 (0.82-2.57)	0.197	7.32 (2.30-23.28)	**0.001**
	No (n=86)	1 (Reference)	-	1 (Reference)	-
Drinking	Yes (n=15)	0.99 (0.47-2.10)	0.984	0.19 (0.02-0.50)	**0.005**
	No (n=92)	1 (Reference)	-	1 (Reference)	-
BMI	≤25 (n=57)	1 (Reference)	-	1 (Reference)	-
	25-30 (n=37)	1.04 (0.61-1.77)	0.899	0.64 (0.32-2.03)	0.639
	≥30 (n=13)	1.11 (0.49-2.53)	0.800	0.71 (0.15-3.39)	0.672

CRC: Colorectal cancer; BMI: Body mass index. HR: Hazard ratio; CI: Confidence interval; N/A: Not applicable. ᵃ Unadjusted hazard ratio; ᵇ Adjusted hazard ratio

The present study demonstrated that not only do *KRAS* codon 12 mutations have a worse OS (Log-rank P=0.049), but they also have a significantly higher hazard of mortality (HR=2.01) than *KRAS* codon 13 mutations (HR=0.58) and wild-type *KRAS* genes. These results are consistent with some previous reports,[9,10,20] and seem to infer that *KRAS* codon 12 mutations have a greater oncogenic capacity that leads to their poor prognosis.[9,10,18,19] There is some theories about the differences in the more aggressive cellular transformation between different point mutations of the *KRAS* gene, especially *KRAS* codon 12 mutations, which are corroborated by *in-vitro* studies.[9,18,19] The present findings are in line with a report from China in which Bai et al revealed similar prognostic effects for *KRAS* codon 12 and 13 mutations with HR values equal to 2.53, and 0.66, respectively.[10]

This study included only the metastatic CRC patients and the most common metastatic site in them was the liver (n=46, 26.6%). These patients had a significantly worse OS (HR=2.49; 95% CI: 1.49-12.52; *P*=0.002). The present findings differed from some previous findings, as some researchers had formerly found that *KRAS* mutations are more associated with metastasis in the lung and less in the liver.[21,22] A large Australian mCRC database analysis revealed that lung metastasis is more frequent in mCRCs with *KRAS* mutations and reported significant adverse outcomes for the brain, bone, and peritoneal metastasis, while liver metastasis did not have any impact on the OS (HR=1.06) and the median OS was significantly higher in the liver-only metastatic CRCs.[4] Another notable finding of the present study was that *KRAS* mutations are a significantly negative prognostic factor in distal colon tumors (HR=3.36) compared to proximal colon cancers, which is in line with a few previous reports,[7,23] but different from some other studies; for instance, Jones et al did not find any significant impact for tumor location on OS.[9] Meanwhile Prasanna et al. also reported that left-side tumors have better survival compared to right-side and rectal tumors.[4] This disparity of findings may be due to certain environmental and genetic alterations in different mutation subtypes and the clinicopathological characteristics of CRC.

Alcohol consumption (any type in any amount and with any frequency) is a known risk factor for CRC, as evidence suggests that long-term alcohol consumption is associated with an approximately 50% increased risk of CRC.[24-26] The present research study did not find any associations between alcohol intake and the prognosis of mCRC, which is in line with the results of some studies[24]; however, one reason could be that alcohol consumption is generally less prevalent in Iran due to cultural and religious restrictions. Recent surveys in Iran have shown that the prevalence of smoking is high in northwestern Iran, and in East Azerbaijan Province, 23.7% of men and 0.7% of women smoke.[27] The present study also showed that smoking is associated with a much lower OS with a

hazard of 7.32 (P=0.001). Javasekara et al, however, found no associations between smoking and CRC-specific survival.[24] The association between BMI and survival in CRC was less clear and the examined BMI subgroups of this study showed no significant associations with the mCRC survival.[24,28] These results may highlight the importance of adding other anthropometric measures in line with BMI to better assess the role of obesity as a known risk factor of at least seven common cancers.[28]

The main limitation of this study was the lack of mutational tests results for all included CRCs, and so lack of a statistical analysis of *NRAS* and *BRAF* mutations, owing to the low percentage of these mutations in the mCRC cases diagnosed, which could not offer a good statistical power. Another limitation of the study was the lack of cancer treatment data, because in most cases, the patients could not access properly to the targeted therapies as per the standard recommended guidelines due to the poor drug availability and the high costs of treatment. Therefore opted for the routine chemotherapy provided by the oncology center.

However, mutations were detected by Idylla RAS mutation tests (for *KRAS/NRAS/BRAF*), in the referral molecular lab, which is a fully automated Real-time PCR-based system, with approved reliability due to the convenience and practicality of mutation detecting. The validation of reliably detecting of RAS mutations using the Idylla system has been confirmed recently, comparing the gold standard method (Sanger sequencing).[29-32]

Conclusion

Some recent research has suggested that the pattern of specific *KRAS* gene mutation, and so the impact of specific amino acid changes, are associated with different prognostic values and responses to anti-*EGFR* therapies. Routinely performing specific oncogene tests, including

KRAS/NRAS/BRAF mutation testing, at all oncology centers may help oncologists make a better treatment decision and may thus improve the patients' prognosis and life expectancy. The present findings clearly demonstrate that metastatic CRC with mutations in codon 12 of the *KRAS* gene has an independent and significantly worse OS. Also, smoking, liver metastasis, and distal colon tumors are associated with poor disease prognosis. Specific mutations of oncogenes involved in the tumorigenesis of CRC may suggest a different aggressiveness and response to treatment.

Ethical statement
The ethics committee of Tabriz University of Medical Sciences has been approved this project, and all patients' information and records are confidential (IR.TBZMED.REC.1395.18).

Competing interests
The authors have no conflicts of interest.

Authors' contribution
RD, SD, and MHS made substantial contributions to the conception and design of the work; and the acquisition, analysis, interpretation of data. MZ made data extraction and management. ATE performed the DNA extraction and genotyping in the laboratory, interpreted the results and the creation of new molecular tests used in the work. RD, SD, FF, and MHS drafted the work or revised it critically for important intellectual content. All authors approved the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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