KRAS mutations and subtyping in colorectal cancer in Jordanian patients

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Abstract. Colorectal cancer (CRC) is one of the most common malignancies in the Western world and Jordan. v-Ki-ras2 Kirsten rat sarcoma (*KRAS)* mutations represent an early event in the development and progression of CRC. Previous studies have demonstrated that *KRAS* mutations serve as a predictor of response to EGFR-targeted therapies for patients with metastatic CRC. The aim of this study was to determine the portion of CRC patients with wild-type *KRAS* status and molecular subtypes of *KRAS* mutations in Jordan as compared with other countries. DNA was isolated from 100 consecutive colorectal carcinoma specimens from patients who underwent surgical resection or colonoscopic biopsies of colorectal tumors and had developed metastatic disease. *KRAS* mutations were detected by hybridization-based strip assay as well as RT-PCR-based assay and confirmed by standard Sanger sequencing of codon 12 and 13 of exon 1 of the *KRAS* gene. Among 100 tested patients, 56% had a wt-*KRAS* genotype and 44% had a mutated *KRAS* genotype. The pGly12Asp was the most commonly detected mutation (54.5%). *KRAS* mutations were independently associated with patient age, gender and tumoral variables. The ratio of mutated versus wt-*KRAS* patients in this study is similar to those reported in Western countries but contrasts to neighboring Middle Eastern countries. Colorectal carcinoma cases from Jordan had higher *KRAS* mutation frequencies compared with other Middle Eastern countries which is likely to reflect different molecular pathogenesis and environmental exposures.

Introduction

Colorectal cancer (CRC) is the second most common form of cancer in developed countries, only surpassed by prostate cancer in men and breast cancer in women (1). In Jordan, it is the most common type of cancer among men and the second most common among women (2). The reasons for this are unknown and may include both genetic and environmental factors. CRC can be cured by relatively simple colorectal procedures if detected early. However, distant metastasis is the main cause of mortality in CRC patients. Studies have shown that depending on the stage of the primary tumor, liver metastases occur in 20-70% of patients and lung metastases occur in 10-20% of patients (3).

Significant advances have been made in the treatment and outcome of CRC over the last decade. An improved understanding of the molecular pathways involved in the development and progression of CRC has made it possible to provide prognoses for patients with metastases, as well as the development of new therapeutic strategies. The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor. It is expressed in epithelial tissues and acts as a cell growth promoter. According to literature, EGFR contributes to the development and progression of several types of cancer, including CRC where it is overexpressed in 50-80% of colorectal tumors, making it a suitable target for anticancer therapies (4). Abnormal activation of the EGFR pathway may be caused by EGFR overexpression or mutational activation of the downstream elements (5).

Currently, two strategies to attenuate EGFR signaling are in use: monoclonal antibodies that bind to the ligand-binding domain and inhibit the binding of specific ligands (cetuximab and panitumumab), or small EGFR tyrosine kinase inhibitor molecules that bind to the intracellular domain of EGFR and compete for binding with ATP, inhibiting tyrosine phosphorylation (gefitinib and erlotinib). These inhibitors of EGFR have emerged as an important treatment for metastatic colorectal cancer (mCRC) (6,7). To optimize the benefits and reduce the risks of anti-EGFR therapies, EGFR as well as the molecules involved in its signaling pathway have been evaluated as potential markers for predicting therapy outcomes. Anti-EGFR therapies are only effective in a subset of patients with CRC. A number of clinical trials have demonstrated that EGFR-targeted therapies are not effective in patients whose tumors have a mutation in the oncogene Kirsten *ras* (*KRAS*) (8-10).

The v-Ki-ras2 Kirsten rat sarcoma (*KRAS*) gene is a member of the Ras gene family that encodes small G proteins with intrinsic GTPase activity. *KRAS* is a downstream component of the EGFR signaling pathway. It acts as an intracellular signal transducer by coupling the signal from the

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cell surface receptors to intracellular targets, which regulate significant functions for tumor progression, including proliferation, differentiation and apoptosis (4). Mutations in the *KRAS* gene (typically point mutations) result in constitutive guanine triphosphotase activity, which continuously activates signaling pathways in the absence of any upstream stimulation of EGFR/HER receptors (11). Thus, patients with *KRAS* mutations have poor responses to therapy with anti-EGFR inhibitors. *KRAS* mutations are thought to be a fairly early event in carcinogenesis and range from 35-45% in CRC (12). *KRAS* mutations also occur frequently in non-small-cell lung and pancreatic carcinomas (13). The most common mutations identified in CRC occur in exon 2 and to a lesser extent in exon 3 (14). Tumors that have a mutation in codon 12 or 13 of the *KRAS* gene will not respond to treatment with EGFR inhibitors, including cetuximab or panitumumab.

The mutation status of the *KRAS* gene provides diagnostic, prognostic and predictive information for several types of cancer. The precise frequency and genotyping of *KRAS* mutations in the Jordanian population have not been determined. The high incidence and mortality among CRC Jordanian patients indicates the need to determine whether specific ethnic, geographical, dietary or lifestyle factors may possibly be correlated. This study investigated the general incidence of *KRAS* mutations in CRC in Jordan and the incidence of specific mutation types. The possible correlation of molecular results with clinical and histopathological data was also analyzed.

Materials and methods

Ethics statement. This was an observational study. All patients were managed in accordance with normal clinical practice. The Institutional Review Board at King Hussein Cancer Center in Amman, Jordan, approved the current study.

Tissue attainment. Colorectal carcinoma specimens from 100 consecutive patients who underwent surgical resection or colonoscopic biopsies of colorectal tumors and had developed metastatic disease were studied. Biopsies or resected tumors were reviewed for their histological diagnosis and quantification of neoplastic cellularity (>20%). Using hematoxylin and eosin-stained slides, areas with >50% tumor cells were delimited.

All tumors were histologically confirmed to be colorectal adenocarcinomas. In addition, medical records were reviewed for information on the tumor site, pathological stage, presence or absence of metastasis and outcome in patients prior to anti-EGFR therapy. The formalin-fixed paraffin-embedded (FFPE) tissues were previously processed according to routine practices.

DNA extraction. DNA was extracted from paraffin blocks that best represented each tumor, previously selected from hematoxylin-eosin stained slides. To prevent cross contamination from tissues with flakes of paraffin, disposable scalpel blades were used. Tumor areas were carefully scraped from tissue blocks by macro-dissection using a sterile scalpel blade and then transferred to a microcentrifuge tube. Tissues were deparaffinized with three baths of xylene for 10 minutes followed by three baths of 100% ethanol solution for 5 minutes. Following this, tissues were digested with Proteinase K and genomic DNA was isolated using the QIAamp DNA Extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

Samples of isolated genomic DNA were analysed by 0.8% agarose gel electrophoresis to evaluate the DNA quality. The DNA quantity was assessed by using NanoDrop 1000 (Thermo Scientific, DE, USA) and the purity was evaluated by calculating the 260/280 ratio.

KRAS mutational analysis. *KRAS* mutations were detected by an hybridization-based strip assay (ViennaLab® Diagnostics GmbH, Vienna, Austria) as well as a real-time PCR-based mutation assay (DxS *KRAS* Mutation Test kit, DxS Ltd; Manchester, UK) according to the manufacturer's instructions. The first assay is based on reverse-hybridization of biotinylated PCR products to a parallel array of allele-specific oligonucleotides immobilized on membrane strips. The detection of specifically bound mutant *KRAS* alleles is visible by an enzymatic color reaction which can be compared to specific controls. This assay detects 10 mutations located in codons 12 and 13. The second assay designed by DxS Diagnostic Innovations, combines allele-specific PCR Amplification Refractory Mutation System (ARMS) with real-time PCR to detect the seven most common mutations at *KRAS* codons 12 and 13 (p.Gly12Ala, p.Gly12Asp, p.Gly12Arg, p.Gly12Cys, p.Gly12Ser, p.Gly12Val and p.Gly13Asp). Mutation detection was performed with a Rotor Gene Q Real-Time PCR System (Corbett Robotics, Brisbane, Australia).

Random samples were selected for confirmation by standard Sanger sequencing using BigDye® terminator v3.1 (Applied Biosystems, Foster City, CA, USA). PCR was performed to amplify codons 12 and 13 of exon 1 in *KRAS* using specific primers under the PCR conditions described previously (15). The efficiency and quality of the amplification PCR were confirmed by running the PCR products on a 2% agarose gel. A negative control containing all the components of the PCR except the template was included in each PCR. DNA amplified products were purified using a QIAquick DNA clean up kit (Qiagen), according to the manufacturer's instructions. Amplification products were subjected to direct sequencing using the same primers and all mutations were confirmed by sequences originating from both the upstream and downstream primers on ABI 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The presence of a mutation was accepted when its chromatographic peak height was 25% or higher than the peak of the wild-type reference.

Statistical differences were analyzed using a student's t-test and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. Specimens from 100 tumors were retrospectively analyzed for the presence of *KRAS* mutations in codons 12 and 13. The study included almost equal numbers of males and females (Table I). The median age at diagnosis was 55 years. The most common metastatic site was the liver (70% of patients). The primary tumor site was the colon NOS (not otherwise specified) in 58% of patients, the rectum in 22% and 20% were considered to be rectosigmoidal. All cancers

Table I. Characteristics of the 100 patients enrolled in the study.

Characteristic	Value
Total number of tumors	100
Gender	
Female	45
Male	55
Median age at diagnosis (range)	55 (22-74 years)
Primary tumor site	
Colon, NOS	58
Rectum	22
Rectosigmoid	20
Metastatic site at diagnosis	
Liver	70
Lung	17
None	13
TNM stage at diagnosis	
1	0
2	5
3	8
4	87

NOS, not otherwise specified.

were adenocarcinomas and were graded according to WHO criteria.

Prevalence of KRAS subtype mutations in Jordan. Of the 100 tumors included in this study, 44% harbored *KRAS* mutations in either codon 12 or 13 (Fig. 1). Of the majority of *KRAS* mutations, 39 (89%) were identified in codon 12, while codon 13 was involved in 5 (11%) tumors (Fig. 2). Of the 39 mutations in codon 12 (wild-type GGT), 25 (62.5%) were transition mutations, of which GAT (55%) was the most common and 15 (37.5%) were transversion mutations, of which GTT (14%) was the most frequent; in codon 13 (wild-type GGC), only GAC transitions were present (Fig. 3). In one tumor, two mutations were identified in codon 12, each with a transition and transversion mutational type (Gly to Asp and Gly to Cys). Several positive samples were randomly selected to confirm the detected mutation(s) by sequencing. A summary of all molecular types of *KRAS* mutations is shown in Table II.

Correlation of molecular findings with clinical and demographic data. *KRAS* mutations (codon 12 or 13) did not show any significant correlation with tumor location, stage, age at onset or gender of the patient (Table III).

KRAS mutations and prognosis. The response to standard therapy was documented for only 51 patients in the KHCC Cancer Registry. This is a small pool of data; however, findings are shared even if statistical analysis was challenging. In the distribution of patients with either wild-type or mutated *KRAS*, there is no association between response to therapy and gene mutational status (Table IV). A complete response was observed in one patient out of 25 with wild-type *KRAS*. Patients (7) with partial responses included 3 *KRAS* wt and 4 *KRAS* mutants. A similar pattern was also observed for patients with stable or progressive disease. Of the patients with the mutated *KRAS* gene, 73% had progressive disease compared with 68% of patients with the wild-type gene.

Prevalence of KRAS mutations in Jordan and other countries. A review of studies published from various countries concerning the prevalence of *KRAS* mutations in colorectal tumors is

Table II. Spectrum of *KRAS* mutations in 100 colorectal cancers.

KRAS subtype mutation	No.	%
pGly12Asp; codon 12 GGT>GAT	24	54.5
pGly12Val; codon 12 GGT>GTT	6	13.6
pGly12Cys; codon 12 GGT>TGT	5	11.4
pGly12Ala; codon 12 GGT>GCT	2	4.5
pGly12Arg; codon 12 GGT>CGT	2	4.5
pGly12Ser; codon 12 GGT>AGT	1	2.3
pGly12Leu; codon 12 GGT>CTT	0	0
pGly12Ile; codon 12 GGT>ATT	0	0
pGly13Asp; codon 13 GGC>GAC	5	11.4
pGly13Cys; codon 13 GGC >TGT	0	0

KRAS, v-Ki-ras2 Kirsten rat sarcoma.

Table III. Correlation between *KRAS* mutational status and tumoral variables.

	Mutated *KRAS*		wt *KRAS*		
Characteristic	No.	%	No.	%	P-value
Gender					1.000
Female	22	50	23	41	
Male	22	50	33	59	
Age (years)					1.000
>60	11	25	22	39	
<60	33	75	34	61	
Tumor location					1.000
Colon, NOS	58	58	20	67	
Rectum	22	22	5	17	
Rectosigmoid	20	20	5	16	
Primary tumor stage					1.000
I	0	0	0	0	
II	4	9	3	5	
III	5	11	11	20	
IV	35	80	42	75	

KRAS, v-Ki-ras2 Kirsten rat sarcoma; NOS, not otherwise specified.

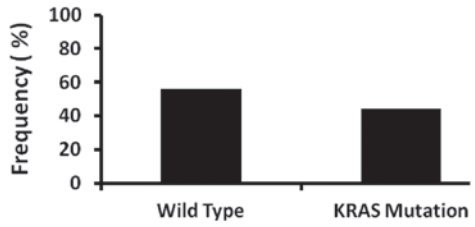

Figure 1. Frequency of *KRAS* mutations among Jordanian CRC patients. *KRAS*, v-Ki-ras2 Kirsten rat sarcoma.

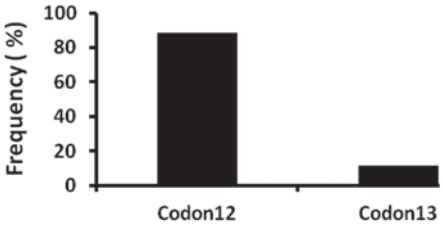

Figure 2. Mutational events in *KRAS* gene. *KRAS* codons 12 and 13 analysis in 100 patients. *KRAS*, v-Ki-ras2 Kirsten rat sarcoma.

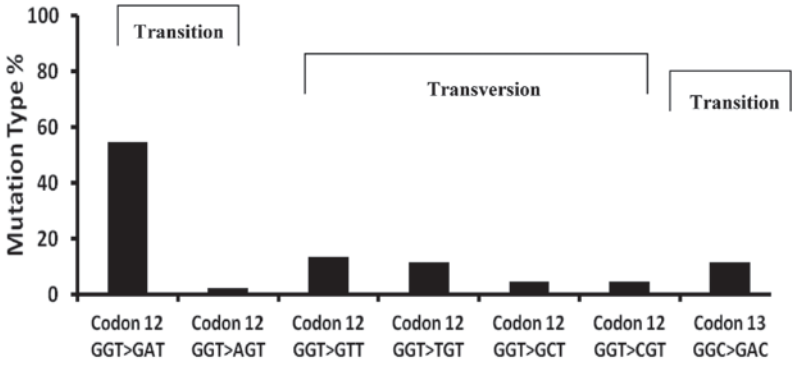

Figure 3. Distribution chart of tested *KRAS* mutations in the group of tested patients. The CRCs of Jordanian patients had more transitional mutations as compared with transversion mutations. *KRAS*, v-Ki-ras2 Kirsten rat sarcoma.

Table IV. Association of *KRAS* mutational status with treatment outcomes.

	Mutated *KRAS*		wt *KRAS*	
Response to therapy	No.	%	No.	%
Complete response	0	0	1	4
Partial response	4	15.5	3	12
Stable disease	3	11.5	4	16
Progressive disease	19	73	17	68

KRAS, v-Ki-ras2 Kirsten rat sarcoma.

shown in Table V (16-21). In most countries, the *KRAS* mutation rate ranged from 18-47%, as identified in Egypt and the United States, respectively. Thus, the prevalence rate identified in Jordan (44%) is at the higher end of this range. In addition, the prevalence of *KRAS* mutations in Jordanian patients was markedly higher than its two closest neighbors, Saudi Arabia and Turkey (28 and 37.5%, respectively). However, the fraction of mutations revealed in codon 12 and 13 of Jordanian CRC patients was similar to all other countries, with the exception of the United States.

Discussion

CRC is one of the leading causes of mortality due to cancer in Jordan. The poor prognosis of this disease would be ameliorated if curative surgery was performed in its early stages. This study analyzed *KRAS* mutations in CRCs of Jordanian patients, in whom CRC incidence and mortality is one of the highest in the Middle East and is still increasing (2). The incidence of *KRAS* mutations in Jordan was 44% and these mutations were predominantly observed in codon 12 (89%). These results, from a series of 100 patients, are in accordance with a modern series conducted worldwide. *KRAS* codon 12 and 13 mutations cover 98% of the entire *KRAS* mutation spectrum in CRC (22,23). Thus, this analysis did not include other codons, including 61 (exon 3) or 146 (exon 4) due to their infrequency in CRC.

To assess the specificity of both the TheraScreen DxS *KRAS* Mutation Kit and Vienna Lab methods used to analyze *KRAS* status in this study, the concordance of test results was evaluated for retrospective samples with the results of the Sanger sequencing method. The real-time PCR results (DxS kit) and allele-specific oligonucleotide hybridization results (Vienna Lab) were in 100% concordance when compared with the Sanger sequencing method. A number of comparative studies have evaluated the performance of the various methods used to accurately characterize *KRAS* gene status (24,25). The majority of these studies agree that the DxS and Vienna Lab kits are equivalent and more reliable due to their higher sensitivity than Sanger sequencing (24). The DxS kit tests for the seven most common mutations in codon 12 and 13 of *KRAS* and Vienna Lab detects 10 mutations. A significant number (44%) of *KRAS* mutations were detected using these two kits which may account for a large number of mutations in the Jordanian population. Thus, the detection methods utilized in this study are considered to produce an accurate frequency of *KRAS* mutations.

Table V. Prevalence of *KRAS* mutations in Jordan and other countries.

	Jordan	Saudi Arabia	Egypt	USA	Brazil	Turkey	Spain	Slovenia
KRAS mutated tumors (%)	44	28	18	47	35	37.5	34	45.5
Mutated in codon 12 (%)	89	81	NA	96	85	82	84	81
Mutated in codon 13 (%)	11	19	NA	4	15	18	16	19

KRAS, v-Ki-ras2 Kirsten rat sarcoma.

The frequency and spectrum of *KRAS* mutations did not differ when compared with those of most other studies (16-21). The distribution of the seven tested mutations (p.Gly12Asp, 54.5%; p.Gly12Val, 13.6%; p.Gly12Cys, 11.4%; p.Gly12Ala, 4.5%; p.Gly12Arg, 4.5%; pGly12Ser, 2.3% and p.Gly13Asp, 11.4%) among the mutated *KRAS* patients is in accordance with published data (21). The findings of this study suggest that the frequency of *KRAS* mutations in Jordan is similar to those in European countries and the United States. However, Jordanian *KRAS* mutation data contrasted sharply with the neighboring countries of Saudi Arabia and Egypt. A study concerning *KRAS* mutation status in Saudi Arabia reported a significantly lower frequency (28%) than its neighboring Jordan (19). An Egyptian study reported that mutations of the *KRAS* proto-oncogene is uncommon (18%) in Egyptian CRC in contrast to Western cases and was not identified in any patients under the age of 40 years (26). Although Arabic countries share certain cultural background and environmental exposures, these findings reveal possible molecular genetic determinants playing a role in *KRAS* gene mutation. Thus, ethnicity and geographical differences should be considered in designing future clinical trials. Overall, the frequency and spectrum of *KRAS* mutations did not differ when compared to the majority of other reports, possibly due to the nature of mutations in the *KRAS* gene giving the tumor cell a growth advantage leading to clonal selection.

In this study, no statistically significant difference between *KRAS* positivity rate and the clinicopathological findings was observed. Certain studies have reported a higher frequency of *KRAS* mutations in females compared with males (18,27). This study had an almost equal ratio of males to females but identified no statistical difference in *KRAS* mutation with respect to gender. Tumor stage revealed no correlation with mutation status, which is in agreement with published studies (17,28).

The association between *KRAS* mutational status and prognosis remains controversial for patients with metastatic CRC that have not been treated with anti-EGFR antibodies. While certain studies reported a link between *KRAS* mutations and poor prognosis (15,17), others have identified no association (29). The biggest clinical trial designed to analyze the prognostic value of *KRAS* status was the RASCAL study, which revealed that a glycine-to-valine mutation in codon 12 increased the risk of recurrence and mortality by 30%, irrespective of the type of therapy administered (29). A smaller scale study from Spain published findings that agreed with the RASCAL study on the poor prognosis for patients with *KRAS*-mutated primary tumors. However, the Spanish group revealed contrasting results to the RASCAL study by declaring that the mutation type did not affect prognosis (17). One limitation in the current study was the unavailability of information concerning treatment outcomes since the study was in retrospect. Only 51 patients had available information concerning response to therapy documented in the KHCC Cancer Registry and of those only 26 were *KRAS*-mutated patients. Thus, we were unable to perform analysis to deduce whether an association existed among disease-free, progression-free or overall survival rates and *KRAS* mutational status or type. However, the available data for the group of patients with stable disease provide inconclusive evidence as the *KRAS* mutational status is negative for almost half of the patients. Additionally, among the 36 patients who were progressing and did not respond to therapy, 17 were *KRAS*-wt and 19 harbored *KRAS* mutations. A similar pattern of results was also reported by Licar *et al* suggesting other molecular elements of response require identification (21).

Information from a larger scale future study in Jordan will either confirm or refute that the presence of activating mutations in the *KRAS* gene reveals a poor prognostic group and non-responding patients to anti-EGFR antibodies. Further investigation will be directed at how Food and Drug Administration agencies handle *KRAS* mutational analysis prior to targeted drug administration to CRC patients.

Increasing efforts are exerted to assess individual specific molecular alterations for personalized diagnosis, prognosis and/or treatment. Future studies should be of larger sizes and any existing concordance between *KRAS* mutations of primary and metastatic tumors from patients with CRC requires identification. A number of previous studies have reported a high degree of concordance in *KRAS* mutational status between primary tumors and their related liver metastases (17,30-32). The liver and lung are common sites of CRC metastases, however, this study revealed liver metastases to be more common (70%). Determining the degree of concordance is critical from three aspects. First, the frequency of *KRAS* mutations in primary and secondary tumors in patients needs to be compared with published studies to determine whether the acquired data are in agreement. Second, evidence is required to further support previous studies that *KRAS* mutations occur early in carcinogenesis (33). Third, evaluation of the mutational status of *KRAS* may be performed from a metastatic site in the case of a primary tumor sample being unavailable.

In summary, *KRAS* mutation and subtyping analysis of CRCs in Jordanian patients confirmed the data from other studies but also yielded potentially new recommendations. The results of this study will have a major impact on disease management as the cost of treating metastatic CRC will be

reduced by millions of Jordanian Dinars a year, if all patients were tested for *KRAS* mutations. Using *KRAS* testing to restrict the use of EGFR-inhibitor therapy to patients with wild-type *KRAS* tumors would avoid the administration of unnecessary, ineffective and toxic treatments to patients with *KRAS* mutations who would not benefit from them.

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