Clinicopathological characteristics and mutational profile of KRAS and NRAS in Tunisian patients with sporadic colorectal cancer

Abstract

Background/aim: Colorectal cancer (CRC) is a major public health problem worldwide and in Tunisia due to its increasing incidence. KRAS and NRAS mutations have become a pivotal part of CRC diagnosis, given their association to treatment resistance with anti-epidermal growth factor receptor (EGFR) monoclonal antibodies. We aim to screen for mutations in KRAS and NRAS genes in Tunisian patients with CRC and to explore their correlations with clinicopathological features.

Materials and methods: AmoyDx KRAS and NRAS mutation real-time PCR kits were used to screen for mutations in KRAS (exon 2) and NRAS (exons 2, 3 and 4) in 96 CRC tumors.

Results: KRAS exon 2 mutations were found in 41.7% (40/96) of the patients. Codon 12 most abundant mutations were G12D and G12V followed by G12A, while G13D is the predominant mutation in codon 13. KRAS exon 2 mutations were associated with older patients (p = 0.029), left-sided tumors (p = 0.037) and greater differentiation (p = 0.044). The prevalence rate of NRAS mutations was 7.3%, mostly in exon 2. These mutations were associated with early stages (p = 0.039) and the absence of lymph node metastasis (p = 0.045).

Conclusion: It can be inferred from this study that Tunisian CRC patients have a similar frequency of KRAS and NRAS mutations compared to those observed in other populations. Consequently, screening for KRAS and NRAS mutation is crucial to orientate the therapies and the selection of an appropriate candidate, avoiding patients’ unnecessary toxicity and costs.
Key words: Sporadic colorectal cancer, KRAS, NRAS, Real-Time PCR, ARMS-PCR,
Tunisia

1. Introduction

Due to its increasing incidence and mortality rates, colorectal cancer (CRC) represents a major health concern throughout the world [1]. This neoplasm ranks as the third most common cancer among both men and women with nearly 1.8 million new diagnosed cases each year [1,2].

The 2018 Global Cancer Statistics ranked CRC as the second leading cause of cancer-related morbidity, with an estimated 881,000 cancer deaths worldwide [1].

As a public health problem worldwide and in Tunisia, CRC incidence increased over the past 20 years [3,4]. In Tunisia, colorectal carcinoma is considered to be the most frequent digestive cancer [5,6].

The adenoma-carcinoma sequence is a multistep process that starts with genetic alterations in early adenoma, of which the accumulation transforms it into carcinoma [7].

Studies pinpointed three major pathways that are responsible for genomic instability in CRC: chromosomal instability, microsatellite instability, and CpG island methylator phenotype [8]. Most colorectal cancers arise through the chromosomal instability pathway due to the accumulation of somatic mutations in proto-oncogene (KRAS) and tumor suppressor genes such as APC and TP53 [8].

KRAS mutations are considered to be an early event in tumorigenesis [8–11]. In colorectal cancer, approximately 30% to 50% of tumors harbor these mutations [11–13]. Approximately 90% of KRAS mutations are located in codons 12 and 13 [11,14]. They
are mostly single nucleotide point mutations, particularly G>A transitions and G>T transversions [15,16].

As a main effector molecule in the epidermal growth factor receptor (EGFR) signaling pathway, mutant KRAS tumors exhibit resistance to EGFR-targeted therapies [17]. Subsequently, the American Society for Clinical Oncology (ASCO) and the National Comprehensive Cancer Network (NCCN) have recommended KRAS gene mutation analysis before anti-EGFR therapies [18,19].

In 2009, the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMEA) deemed the two EGFR antagonists, cetuximab and panitumumab as “not recommended” for the treatment of patients with metastatic CRC (mCRC) harboring KRAS mutations [16,20].

However, most patients with KRAS codons 12/13 wild-type colorectal cancer still fail to respond to anti-EGFR therapy, suggesting the involvement of other mutations [21,22]. In this context, NRAS, a member of the RAS family, is found to be mutated in 1-7% of colorectal cancer [23]. In fact, recent researches showcased that mutations in KRAS exons 3 and 4 and NRAS gene exons 2, 3 and 4 are associated with resistance to anti-EGFR antibody or poor prognosis in mCRC [24,25]. Thus EMEA and ASCO, made it mandatory to investigate exons 2, 3 and 4 of both KRAS and NRAS prior to the use of any novel targeted therapies such as anti-EGFR treatments [2,26].

In conclusion, the RAS gene family (KRAS and NRAS) status allows the orientation of therapies and therefore, make possible to avoid patients’ unnecessary toxicity and costs [8,16,22].
In view of these points, our work aims to screen for mutations in KRAS and NRAS genes in Tunisian patients with sporadic colorectal cancer and to explore their correlations with clinicopathological features.

2. Materials and methods

2.1. Patients and tumor samples

We conducted a retrospective study from 2010 to 2018 regarding 96 sporadic CRC patients. This study protocol was approved by the Ethics Committee of Mongi Slim Hospital, La Marsa, Tunisia.

Mutational analyses were performed on frozen specimens taken from patients who underwent colorectal tumor resection at the Department of Surgery, and on archival paraffin-embedded tissue blocks, either on primary or metastatic samples, preserved at the Department of Pathology and Cytology of the Mongi Slim Hospital, La Marsa, Tunisia.

The clinicopathological features (age, sex, tumor location, histological type, differentiation, depth of invasion, TNM (tumor, node, metastasis) stage and lymph node metastasis) were collected for each patient from surgical and pathological records.

2.2. Samples selection and DNA extraction

After evaluating standard hematoxylin/eosin-stained slides from primary and metastatic colorectal adenocarcinomas, appropriate samples were specifically selected by a pathologist to include predominately tumor cells without significant necrosis or inflammation. Five 5.0-μm-thick unstained sections were cut from the pre-selected paraffin blocks, and for the frozen specimens 25 mg was taken from each sample.
DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen™, Carlsbad, CA, USA) following the manufacturer’s instructions. The DNA concentration was assessed using the Qubit dsDNA HS (high sensitivity) Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) on a Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer’s instructions.

2.3. Analysis of KRAS and NRAS gene mutations by Amplification-Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR)

The AmoyDx® KRAS Seven Mutations Detection and NRAS Mutations Detection kits (both from Amoy Diagnostics Co., Ltd., Xiamen, China) were used to detect KRAS and NRAS status of each DNA sample. Both kits are Chinese Food and Drug Administration (CFDA) approved for clinical use in China and Conformite Europeenne (CE) marked for in vitro diagnostic (IVD) use in Europe.

These highly sensitive kits are based on a patented technology ADxARMS, enabling the detection of 1% mutant DNA in a background of 99% normal DNA at 10 ng DNA sample, while ensuring minimal false negatives.

The AmoyDx® KRAS Seven Mutations Detection Kit is designed to accurately identify the 7 most common activating KRAS mutations in codons 12 and 13 (Table 1).

The AmoyDx® NRAS Mutation Detection Kit intended to detect meticulously 16 hotspot somatic mutations in codons 12, 13, 59, 61, 117 and 146 of NRAS gene (Table 1).

The extracted DNA quality was evaluated by amplifying a housekeeping gene and using the HEX channel provided with the kit.
PCR reactions were performed using a Stratagene Mx3005P™ (Agilent Technologies, Inc., Santa Clara, CA, USA) under the following conditions: 5 min incubation at 95 °C, followed by 15 cycles of 95 °C for 25 sec, 64 °C for 20 sec, 72 °C for 20 sec and then 31 cycles of 93 °C for 25 sec, 60 °C for 35 sec, 72 °C for 20 sec. The fluorescent signal was collected from FAM and HEX channels. Note that every PCR run must contain one PC (Positive control) and one NTC (No template control).

KRAS and NRAS mutation status was determined according to the Ct value as indicated in the manufacturer’s instructions.

2.4. Statistical analysis

All statistical analyses were performed using SPSS software V20 (SPSS, Inc., Chicago, IL, USA). Associations between variables were tested with the chi-square ($\chi^2$) test. A probability (P) value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Patient and Tumor Characteristics

In our 96 CRC patients, CRC prevalence was higher in males (62.5%, 60/96) than in females (37.5%, 36/96). The mean age of the Tunisian patients, at tumor resection, was 62.4 years, ranging from 23 to 92 years.

Regarding the histological subtypes of our series, 69.8% (67/96) of tumors were non-mucinous (NMC), and 30.2% (29/96) were mucinous adenocarcinomas (MC).

The tumors were graded according to the WHO criteria (World Human Organization Classification of Tumours of the Digestive System, Fourth Edition) [27] as follows: 47 (49%) were well-differentiated, 46 (47.9%) were moderately differentiated and 3 (3.1%) were poorly differentiated.
A total of 71 samples (73.96%) were located in the left colon and 25 (26.04%) in the right colon.

Histologic classification of tumors was made according to the international TNM staging system based on the eighth edition of the American Joint Committee on Cancer (AJCC-8th edition) [28].

Specimens were taken from 70 primary tumors (72.92%) and 26 metastasis (27.08%), distributed between 32 cases in the primary stage (stages I and II) and 64 cases in advanced stage (stages III and IV).

### 3.2. Distribution of KRAS and NRAS mutations in colorectal carcinomas

The distribution of KRAS and NRAS mutations in the 96 CRC patient samples is presented in Table 2.

KRAS exon 2 mutations were observed in 41.7% (40/96) of the cases. Note that 7 cases had two concomitant mutations. So in a total of 40 cases, we had 47 mutations in KRAS exon 2 distributed as follows: 40 (85%) were detected in codon 12, and 7 (15%) were identified in codon 13.

The most prevalent mutations were G12D and G12V 25.5% (12/47) each, followed by G12A 17% (8/47), then G13D 14.9% (7/47), G12R and G12C 6.4% (3/47) each, and G12S 4.3% (2/47).

Mutations outside KRAS exon 2 were observed in NRAS gene, in 7.3% (7/96) of the cases. One case showed two NRAS mutations. These 8 mutations were distributed as follows: 62.5% (5/8) in exon 2 (codons 12 or 13), 37.5% (3/8) in exon 3 (codon 61), and none in exon 4 (codon 146).

The Figure displays the different mutation profiles as shown in sub-figures (a), (b), and (c).
In our study, none of the patients harbored a simultaneous mutation in \textit{KRAS} (exon 2) and \textit{NRAS} (exons 2, 3 and 4). Therefore, these mutations were mutually exclusive.

3.3. Association between \textit{KRAS}/\textit{NRAS} Mutations and Clinicopathological Features

A summary of the relationships between \textit{KRAS} and \textit{NRAS} mutations and various clinicopathological features is provided in Table 3. \textit{KRAS} mutations were much higher in older patients (>60 years old) (80% vs. 20%, \(p = 0.029\)) and were significantly more prevalent in the left side of the colon than the ones on the right side (85% vs. 15% respectively; \(p = 0.037\)). Meanwhile, these mutations were significantly associated with well-differentiated tumors but less with moderately and poorly differentiated tumors (62.5% vs 37.5% vs 0% respectively; \(p = 0.044\)). Although \textit{KRAS} mutations frequency is higher in NMC (80%) the difference is not statistically significant (\(p = 0.066\)).

There was no significant relationship between \textit{KRAS} mutations and sex (\(p = 0.669\)), lymph node metastasis (\(p = 0.446\)) or tumor stage (\(p = 0.884\)). \textit{NRAS} mutations were more frequent in stage I and II (71.4%) compared with stage III and IV cancers (28, 6%) (\(p = 0.039\)), and were associated with the absence of lymph node metastasis N0 (\(p = 0.045\)). However, no significant relationship was observed between \textit{NRAS} mutations and sex (\(p = 0.184\)), Age (\(p = 0.149\)), tumor location (\(p = 0.260\)), histological type (\(p = 0.120\)) or tumor differentiation (\(p = 0.151\)).

4. Discussion

As the third most common cancer among men and women, CRC has increased in terms of incidence and mortality worldwide and in Tunisia [1,3].
The 2018 Global Cancer Statistics ranked CRC as the second leading cause of cancer-related morbidity, with an estimated 881,000 cancer deaths worldwide and nearly 1.8 million new diagnosed cases each year [1,5,6].

It has been widely established that the KRAS mutation pattern has a significant impact on the orientation of anticancer therapy. In this context, tumors harboring exon 2 KRAS mutations (codons 12 and 13) do not benefit from EGFR targeted therapies. Interestingly, some wild-type KRAS exon 2 patients did not respond well to anti-EGFR therapy proving that additional RAS mutations (KRAS exons 3 and 4 or NRAS exons 2, 3 and 4) can negatively predict the success of anti-EGFR treatment.

In the present study, the frequencies of KRAS and NRAS gene mutations were determined in Tunisian patients with sporadic CRC. Additionally, we investigated correlations between these genetic mutations and clinicopathological features. Our results are consistent with previous studies where 50% of colorectal cancers harbored a RAS mutation [29]. With a KRAS exon 2 mutation frequency of 41.7%, we were in accordance with Tunisian and worldwide studies where frequencies ranged respectively from 15% to 46% [30–33] and from 30 to 50% (summarized in Table 4).

Therefore, we noticed that KRAS mutations arise at similar frequencies in Tunisian patients as in other populations, this may be attributed to the involvement of the same genes in sporadic colorectal carcinomas, regardless of the variation imposed by ethnicity, geographical distribution, dietary, lifestyle factors and sensitivity of the different techniques used in previous studies.

In accordance with preceding reports, 90% of KRAS mutations found in our cohort are located in codons 12 and 13, where the majority occurred in codon 12 (85%) and (15%)
in codon 13 and most frequently observed types of mutations are G>A transitions and G>T transversions \([14,16,31,34–36]\).

We found that the most abundant mutations of codon 12 were G12D and G12V while G13D is the predominant mutation in codon 13. These results are concordant with the local Tunisian studies \([30,31,33,37]\) and the international ones \([12,34,36,38–41]\).

Similar to literature data, we also report a cluster of four mutation types (G12D, G12V, G12A, and G13D), which accounts for 84.8\% (39/46) of KRAS exon 2 mutations \([38,39,41]\).

Correlations between KRAS and NRAS mutational status and the different clinicopathological features are very controversial. Some previous reports pinpointed that the frequency of KRAS and NRAS mutation was associated with various clinicopathological criteria, but others did not.

Regarding KRAS exon 2, most of our results were consistent with the literature, notably the association with age, tumor location and histology.

Our data showcased that KRAS exon 2 mutations seemed to occur frequently in elderly patients. This result was supported by many other studies \([20,41–43]\).

When it comes to tumor location, disparities have been reported where KRAS mutation rates were higher in the right-sided CRC tumors \([2,36,40,44]\). Our study, along with others, showed an association rather with the left side of the colon \([45–48]\).

The cause of the divergent findings between left and right side colon adenocarcinoma are still unclear \([40]\). It could be attributed to the complex origin and the exposure of left-sided luminal microenvironment to ingested carcinogens and mutagens \([40]\).

Our data align with the ones reported in literature where KRAS mutations showed a significant association with well-differentiated tumors but less with moderately
differentiated tumors, with no or few KRAS mutation, found in poorly differentiated
tumors [2,29,31,35,36,46,48].

Association between KRAS mutation and mucinous histotype was reported in some
studies [36,46,49] but denied in others [29,50,51], including ours.

Unlike KRAS mutations that are strongly implicated in colorectal cancer, NRAS
alterations are rare and, to date, little data on its mutation prevalence are available [36].

In our study, the NRAS mutation rate was 7.3%, similar to the only available Tunisian
study which reported 6.9% [37].

Our data shows that 12.5% of wild-type KRAS exon 2 patients carried a mutation in
NRAS exons 2 and 3. Furthermore, recent data showed that 12–17% of patients, with
wild-type KRAS exon 2 (codons 12/13), harbor a mutation in KRAS exons 3 and 4 and
NRAS exons 2, 3 and 4 [25,52].

We note that NRAS mutation incidence varied depending on the population where
Zhang reported a rate of 3.69% in a Chinese study [49], whereas a 6% and 6.3%
frequencies were described in Italian and Indian studies, respectively [52,53] versus
9.57% in Greek and Romanian patients [22].

In our study, NRAS mutations were associated with early stages and the absence of
lymph node metastasis.

Some studies have observed that NRAS mutations tended to occur in left-sided cancers
and in women [54]. While Russo et al, reported associations with rectal cancer, and with
age 56 years or older [55].

Chang noted a correlation with the male gender [12]. Furthermore, Shen observed that
these mutations were more frequent in distant metastasis tumors, and its rate varied with
the different tumor stages [39]. Other studies did not find any correlations [29,36,37,49].

This divergence may be attributed to diverse ethnicities, genetic factors, geographical distributions, and diagnostic techniques.

Nowadays, various techniques for assessing RAS mutation status are available, such as Sanger sequencing, high-resolution melt analysis, pyrosequencing and next-generation sequencing techniques [56]. Though the tests varied in terms of sensitivity and specificity, no standard method has yet been endorsed for clinical practice [57].

In our study, we used AmoyDx Mutation Detection Kit, a relatively simple real-time PCR assay, fast and less prone to external contamination [58]. It’s considered to be one of the most sensitive methods available in clinical molecular laboratories [59].

Due to its high sensitivity and accuracy, the AmoyDx KRAS real-time PCR kit has significantly higher mutation detection rates than Sanger DNA sequencing [58]. Therefore, AmoyDx real-time PCR is an effective and reliable tool for clinical screening of somatic gene mutations in tumors such as colorectal [58].

However, we have to point out that the present study was retrospective with small sample size, and focused only on KRAS exon 2 and NRAS exons 2, 3 and 4, preventing us from drawing any firm conclusions.

Our database did not include any detailed information about adjuvant chemotherapy; hence the patients’ adjuvant treatment was not analyzed in the current study.

5. Conclusions

In conclusion, we studied mutations of KRAS and NRAS genes in Tunisian CRC patients and their correlations with clinicopathological features. Our results show that in terms of incidence, KRAS and NRAS mutations occur at similar frequencies in Tunisian
patients as in other populations. Meanwhile, clinicopathological features analysis showed both similarities and differences when contrasted to those reported in other studies.

Consistent with the literature, KRAS exon 2 was associated with older patients, left-sided tumors, and greater differentiation. Otherwise, no association was found with other clinicopathological criteria such as gender, lymph node metastasis, tumor stage, or histological type.

As regards NRAS mutations, our study showcased an association with early stages and the absence of lymph node metastasis; differently with various researches reporting an association with other features like tumor location, gender, or age.

Therefore, screening for KRAS and NRAS mutation is crucial to guide therapies and selection of appropriate candidates, avoiding unnecessary toxicity and costs for patients.

Given the importance of such molecular analysis, future studies can be focused on the evaluation of other biomarkers, suggested of being associated with poor or no benefit from anti-EGFR therapy, such as KRAS exons 3 or 4, or BRAF.

Acknowledgement/Disclaimers/Conflict of interest

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The authors declare that they have no conflict of interest.

References


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<th>Mutation</th>
<th>Base</th>
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**Table 1:** KRAS and NRAS Mutations detected with AmoyDx® kit
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Table 2: Distribution of KRAS and NRAS mutations in the 96 CRC patient samples
**Clinicopathological features** | Number | **KRAS status** | **NRAS status** | P value | P value |
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<td>36 (64.3)</td>
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<td>25 (62.5)</td>
<td></td>
<td>45 (50.6)</td>
</tr>
<tr>
<td>Moderate (n = 46)</td>
<td>46</td>
<td>31 (55.4)</td>
<td>15 (37.5)</td>
<td><strong>0.044</strong></td>
<td>42 (47.2)</td>
</tr>
<tr>
<td>Poor (n = 3)</td>
<td>3</td>
<td>3 (5.3)</td>
<td>0 (0)</td>
<td></td>
<td>2 (2.2)</td>
</tr>
<tr>
<td>Stages</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I, II (n = 32)</td>
<td>32</td>
<td>19 (33.9)</td>
<td>13 (32.5)</td>
<td>0.884</td>
<td>27 (30.3)</td>
</tr>
<tr>
<td>II, IV (n = 64)</td>
<td>64</td>
<td>37 (66.1)</td>
<td>27 (67.5)</td>
<td></td>
<td>62 (69.7)</td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (n = 33)</td>
<td>33</td>
<td>21 (37.5)</td>
<td>12 (30)</td>
<td>0.446</td>
<td>28 (31.5)</td>
</tr>
<tr>
<td>Yes (n = 63)</td>
<td>63</td>
<td>35 (62.5)</td>
<td>28 (70)</td>
<td></td>
<td>61 (68.5)</td>
</tr>
</tbody>
</table>

1 **Table 3**: Correlation between KRAS/ NRAS mutations and clinicopathological features
<table>
<thead>
<tr>
<th>Country</th>
<th>KRAS Mutation Frequency</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Australia</td>
<td>41.6%</td>
<td>[17]</td>
</tr>
<tr>
<td>China</td>
<td>47.2%</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>42.56%</td>
<td>[49]</td>
</tr>
<tr>
<td>France</td>
<td>39.6%</td>
<td>[29]</td>
</tr>
<tr>
<td>Greece</td>
<td>41.3%</td>
<td>[22]</td>
</tr>
<tr>
<td>India</td>
<td>35.7%</td>
<td>[53]</td>
</tr>
<tr>
<td>Italy</td>
<td>50%</td>
<td>[52]</td>
</tr>
<tr>
<td>Romania</td>
<td>39.2%</td>
<td>[22]</td>
</tr>
<tr>
<td>USA</td>
<td>36.2%</td>
<td>[44]</td>
</tr>
</tbody>
</table>

1 Table 4: KRAS mutation frequencies in different countries
Figure: Amplification plots of: (a) Wild-type sample, (b) Sample with one mutation, (c) Sample with two mutations.