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Original article

KRAS Testing for Colorectal Cancer Patients: Our Laboratory Experience in The Libyan National Cancer Institute

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Abstract

Colorectal cancers frequently have activating point mutations in the Kirsten rat sarcoma viral oncogene homolog (KRAS). Nowadays, mutation testing for KRAS gene is recommended before starting anti-epidermal growth factor receptor (anti-EGFR) therapy. KRAS mutations that are constitutively activated are displaying clear linkages to resistance to treatment with anti–EGFR). This test is regularly demanded for patients with colorectal malignancies in order to supply information that can be used for allocating the best anticancer chemotherapy to each individual case. Also, anti-EGFR therapies are expensive and can harm people who don't have activating mutations. In clinical practice, this step of KRAS testing has a crucial consideration because the test's results will influence on decision taking that have implications on treatment outcomes. Variety of techniques are in use to perform KRAS mutation testing worldwide. The aim of this article is to discuss the KRAS mutation analysis approach which is in use at department of histopathology/ national cancer institute (NCI) of Misrata.

Keywords: *Colorectal Cancers, KRAS Mutation, KRAS Testing Techniques.*

Introduction

KRAS mutations appear in several human malignant tumours such as pancreatic, thyroid, colorectal cancers (CRC) and lung with approximate percentages of 90%, 60%, 43% and 30% respectively [1-3]. The CRC is the 3rd most common cancer entity and 4th most prevalent reason of death due to cancer worldwide [4,5]. KRAS gene was first acknowledged as the causative factor of leukemia virus-induced sarcoma in rats [6]. Subsequently, the retroviral oncogene sequence of Kirsten rat sarcoma was cloned and exploited to detect the human homolog gene, currently so called KRAS or KRAS2 (Kirsten rat sarcoma virus 2 homolog) [7, 8]. Currently, a considerable quantity of information has been produced on the role of KRAS gene in normal and cancerous cells, and KRAS sequence analysis became a regular practice of patient management. Usually, KRAS has a main role in cell-signaling pathways [9-12]. Binding between activated wild-type KRAS and GTP leads to a change in its conformation permitting the protein to bind and initiate a group of downstream effectors such as Raf, Braf, mTOR, MEK1 and 2, ERK, AKT, and PIK3CA. Several different effects occur as a result of activation of these downstream effectors, including apoptosis suppression, promotion of cell growth, cell transformation, angiogenesis, migration, and differentiation [9-14]. At molecular level, KRAS works as double switch that interchanges between two states with different molecular conformation; a GTP-bound “active” state and a GDP-bound “off” state. KRAS is positioned at 12p12.1, distances about 38 kb, and encodes a 188–amino acid residue with a molecular weight of 21.6 kDa. These activating mutations in KRAS are point mutations and involves to large extent the KRAS amino acid residues number 12, 13, and 61, leading to constitutive activation of KRAS.

Anti-EGFR therapies are often used for locally advanced or metastatic CRC (mCRC) that are unresponsive to previous treatments with one or more chemotherapeutic regimens. It has been reported that activating KRAS mutations are significantly linked with a resistance to therapies using anti-EGFR [15-19]. Positive results (improved survival) being remarked

only in patients whose tumours express wildtype KRAS [16, 19-23]. For this reason, screening of KRAS mutations, which are detected in around 40 % of CRC, has become routine in the management of metastatic CRC before treatment with anti-EGFR [24, 25]. Therefore, in 2009 the American Society of Clinical Oncology (ASCO) and the National Comprehensive Cancer Network (NCCN) recommended checking for KRAS mutations on all patients with CRC being contemplated as candidates for anti-EGFR therapy [26, 27]. Remarkably, existing criterions concerning testing of oncogenic Ras mutation in mCRC required by the U.S Food and Drug administration (FDA) necessitate identifying the KRAS status by an FDA approved test. The European Medical Agency (EMA) requires the use of approved techniques by a qualified test centre [15].

Several techniques are now available for KRAS mutation testing includes numerous PCR-derived and sequencing-based methods. Remarkably, majority of the tests already established for identification of KRAS mutation emphasis on the hotspot mutations afflicting codons 12 and 13, which constitute >95 % of Ras mutations in CRC [25]. Comparative assessments have been made frequently of the strengths and shortcomings of chosen techniques [28-33], however, apart from the guideline of FDA, no consensus has been reached on the method of choice to examine the status KRAS at molecular level in regular pathological diagnostics [34]. In view of the increase in number of CRC cases that leads to strong request for KRAS mutation testing, an ultimate diagnostic analysis for this goal should not only be appropriately sensitive and specific, but should also be time- and cost-effective for socio-economic reasons.

In Libya, Clinical KRAS mutation testing is now being conducted routinely on CRC cases at Misrata cancer centre, one of only two cancer centres offering this crucial service, that are covering a considerable geographic area due to the increasing demand of this test by both medical and surgical oncologists. Here, we present our experience about performance of KRAS testing procedure and also, we will review the current molecular technique used for this purpose at the National Cancer Institute (NCI) in Misrata-Libya. In Libyan hospitals and clinics, the KRAS testing for a colorectal cancer patient is usually requested by an oncologist. Characteristically, colorectal cancer patients subjecting to KRAS testing are those diagnosed with advanced-stage malignancies necessitating adjuvant therapy. Special form for ordering KRAS testing was prepared by the scientific committee in histopathology department/NCI and distributed to different cancer centres and hospitals throughout the country. Also, it is made available online so can be easily obtained. This form that needs to be filled with the treating oncologist contains the necessary demographic information and important and clinical data related to the disease.

Materials and Methods

Tissue samples of 79 cases of colorectal cancer were analysed for KRAS mutations. The sex, age, site and KRAS genotypes identified in colorectal cancer patients referred from all regions of Libya to the pathology department at national cancer institute (NCI) of Misrata city for KRAS mutation analysis. Idylla™ KRAS Mutation system has been used to perform this test according to the manufacturer's protocol. Currently, at the NCI of Misrata-Libya KRAS mutation testing is conducted on a representative sample of tumour tissue obtained from a patient either for biopsy purpose or after the surgical resection of the cancer. In case of metastasis, consideration must be given to elucidate that the representative tissue sample required for KRAS testing is not obtained from the primary cancer but instead from the secondary cancer.

Sample preparation

DNA needed for KRAS mutation testing is regularly extracted from formalin fixed paraffin-embedded (FFPE) tissue of colorectal cancer. This step is facilitated by sectioning the blocks of colorectal tumours into thin sections of three mm. It is important to select a tumour section with optimum quality to avoid invalid results of the analysis. This achieved through performing the following points: 1) Assessment of the tissue block to make sure that it is free of any substantial defects. 2) The paraffin tissue block sectioned and evaluated microscopically by a proficient pathologist to identify exactly where the tumour-rich areas are versus tumour-poor areas Fig. 1A and1B. 3) The tumour-most rich parts are identified and highlighted with a marker Fig. 1C to exclude necrotic tissue and benign parts of tumoural lesion. Areas with enriched tumour are often have a histology that can be comparatively recognized without difficulty from tumour-free tissue. The selected area/areas of tumour tissue may be simply cut out from the tissue block using a hand tissue puncher as shown in figure 2, or as an alternative way may be scraped off from an unstained slide with a surgical blade or an instrument with sharp edge and then used for KRAS analysis purpose.

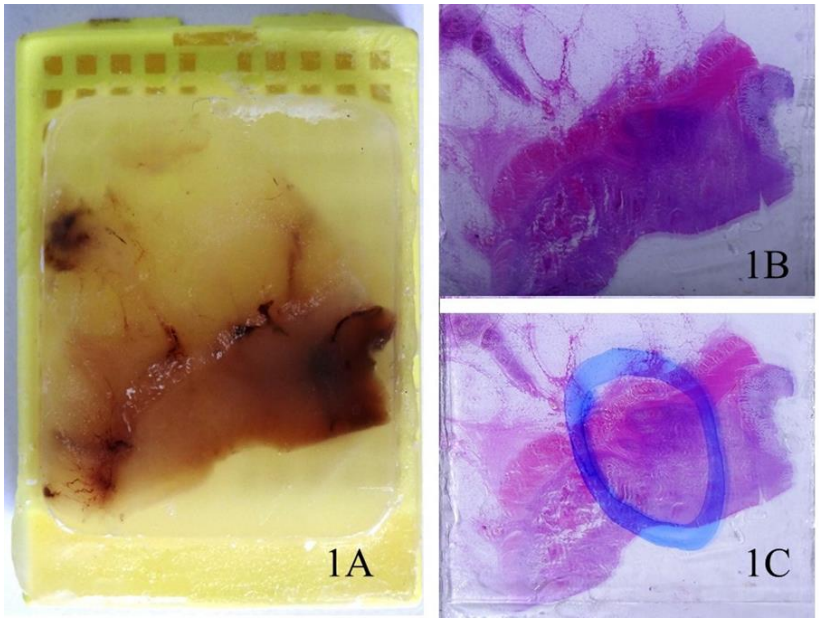

Figure 1. (A) A block of formalin fixed paraffin-embedded (FFPE) tissue of colorectal tumour with good tissue quality. (B) FFPE tissue section with an average thickness of 3mm stained with Haematoxylin & Eosin stain. (C) Via microscopic examination of the Haematoxylin & Eosin tissue section, the tumour-most rich parts are identified and highlighted with a marker.

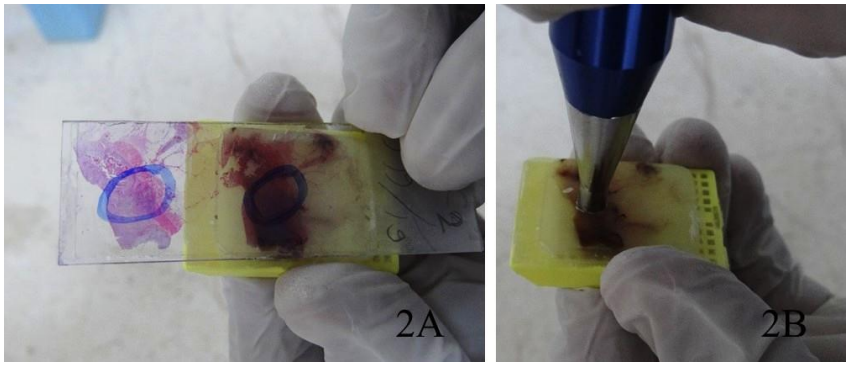

Figure 2. The corresponding paraffin block is used to extract the selected tumour-enriched areas. The selected area/areas of tumour tissue (highlighted area) may be simply cut out from the tissue block using a hand tissue puncher.

Testing Method of KRAS status

Here, we are presenting the KRAS testing technique using the Idylla™ system (Biocartis, Mechelen, Belgium) that is in practice in the two KRAS testing centres in Libya and discuss its different features including sensitivities, specificities, and expenses. The Idylla™ KRAS Mutation Assay allows detection of mutation in three exons named 2, 3, and 4 of the KRAS oncogene. This test performs a group of five allele-specific multiplex PCR reactions, developed for the specific amplification of KRAS gene sequences that are including a mutation in codons 12, 13, 59, 61, 117, or 146 [19]. Thus, this assay can detect twenty-one mutations in KRAS oncogene as the following: seven mutations in exon 2 (codons 12 and 13), nine mutations in exon 3 (codons 59 and 61), and five mutations in exon 4 (codons 117 and 146). In the event of several mutations are present, just the predominantly identified change, lowest ΔCq value, is given [19].

The Idylla™ instrument is fully automated machine and its use to carry out the assay does not necessitate a pre-treatment of the sample such as manual dewaxing of paraffin or FFPE pre-processing (Figure 3).

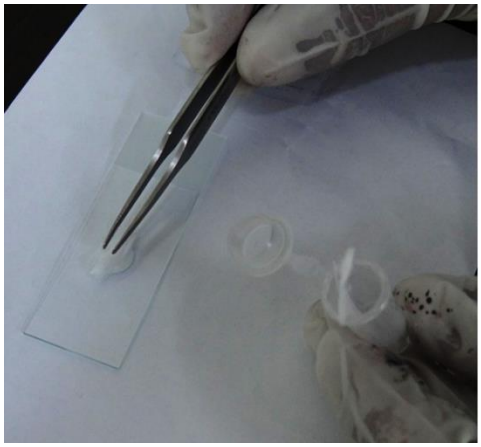

Figure 3. Placement of tissue samples inside the recommended vial tube without need for a manual dewaxing of paraffin prior to processing by the device.

As the Idylla™ instrument is fully automated machine and use closed system, The process of KRAS mutation detection is passing through a group of steps (Figure 4): the FFPE samples were inserted into the cartridge of Idylla™ platform, after that the cartridge is placed into the device where the sample is exposed to a series of reagents, enzymes, heat, and high intensity focused ultrasound (HIFU) resulting in dewaxing of the sample and finally rupture of the tissue and break down of the cells that leads to release of the nucleic acids for following real-time PCR amplification [19]. In each of the five multiplex PCR reactions, a sample processing control, including the concurrent amplification of a monitored section in the junctional region of intron 4/exon 5 of the KRAS gene, was conducted to verify for appropriate carrying out of the entire sample to-result process and as a gauge to assess the availability of the amplifiable amount of DNA in the used tissue sample [19].

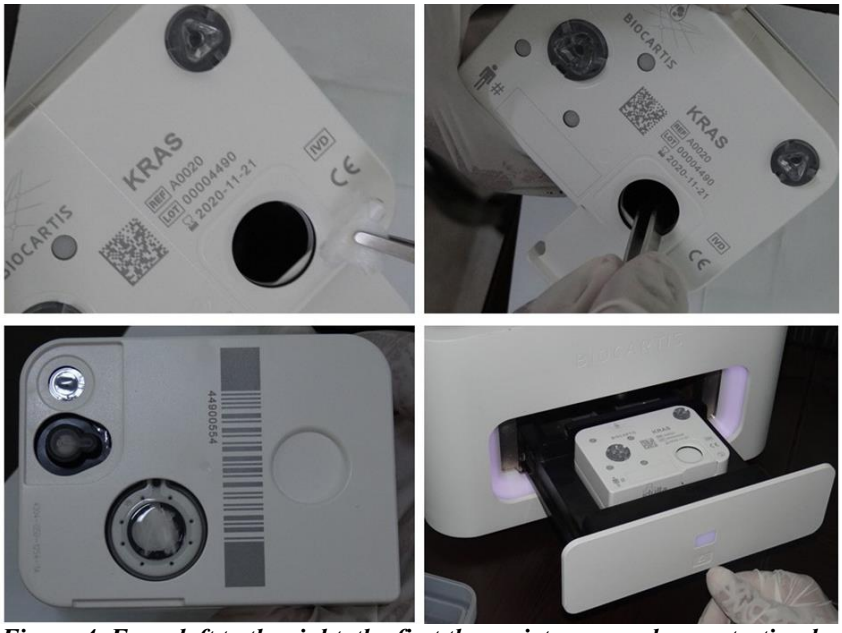

Figure 4. From left to the right, the first three pictures are demonstrating how the FFPE sample is placed inside the cartridge of Idylla™ platform. The last panel shows insertion of the cartridge into the device.

A quantification cycle (Cq) value is calculated by the IdyllaTM software in each successful PCR curve. The KRAS mutation status is regarded as positive if the difference between the calculated Cq for a KRAS mutant PCR signal and the KRAS wildtype Cq value, the ΔCq value, is within a range of approved values and consequently the specific mutation or mutation group is reported. On the other hand, in case the used tissue sample showing a valid KRAS wild-type signal but a ΔCq value outside the validated range is characterised as being negative (wild-type) for KRAS mutation [19]. Invalid results appear as a consequence of several causes such as the improper sample insertion inside the cartridge, inappropriate size of the tissue sample, presence of inhibitors in the sample or inadequate amplifiable DNA. Other factors related to the cartridges themselves can cause invalid results including

incorrect storage of cartridges, use of cartridges that surpassed their allowed period to use after removal from their coverages, or defected cartridges [19].

BRAF and NRAS Testing

Approximately 4.7% and 2.6% of CRC cases may also show BRAF and NRAS mutations respectively [35]. Strong connection also has been found between activating mutation of these genes and a resistance to therapies with anti EGFR [35, 36]. The National Comprehensive Cancer Network therefore suggests checking for BRAF mutations on all patients with CRC being contemplated as candidates for anti-EGFR therapy [27]. The approach used for KRAS mutations testing discussed here are also widely employed for testing on BRAF. Testing for NRAS mutation is not recommended at the current time by the National Comprehensive Cancer Network, although several molecular diagnostic companies such as Biocartis offer this type of tests as shown in Figure 5.

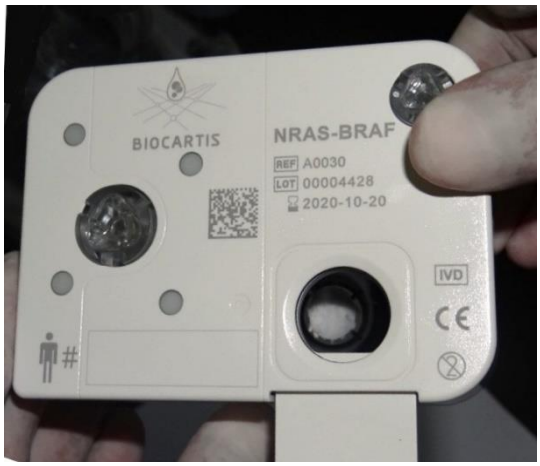

Figure 5. Showing a cartridge of Biocartis system loaded with FFPE tumour tissue for NRAS and BRAF mutation testing using the same approach applied for KRAS mutations testing.

Statistical analysis

All data were tabulated, and statistical analyses were performed in regards to the KRAS genotype and three clinicopathological variables namely age groups, gender and site using the Statistical Package for Social Sciences (SPSS) for Windows version 22.0 (SPSS statistics 22). The frequencies and statistics for the different parameters were studied by using a descriptive analysis.

Results

Tissue samples of 79 cases of colorectal cancer were analysed for KRAS mutations. Of these, 44 (55.7%) reported positive. The remaining 35 (44.3%) samples reported absence of mutation. Overall, there were 39 (49.4%) males and 40 (50.6%) females. Among the KRAS positive cases, there were 23 (52.3%) males and 21 (47.7%) females. Their ages ranged from 32 to 70 years with a median age of 51.14 years. There were 34 patients (77.0%) with point mutations in codon 12 while 3 (7%) had a single mutation in codon 13. There were 3 patients showed mutation in codon 61 with two nucleotide changes whereas the last 4 patients exhibited three nucleotide changes in codon 146. The most common KRAS mutation was p. Gly12Val (c.35G>T) (29.5%), followed by p. Gly12Asp (c.35G>A) (25%). The distribution of the different KRAS mutations identified in Libyan CRC patients is shown in Table 1. The G>A transitions in both codons 12 and 13 accounted for 41.0% of all the mutant KRAS cases. However, the transversions G>T in codon 12 alone forms 38.6 of the total KRAS mutation.

Table 1. Distribution of the different KRAS mutations identified in Libyan CRC patients.

KRAS mutation type	Number of patients	Percentage
Codon 12		
p.Gly12Ala (c.35G>C)	2	4.5%
p.Gly12Cys (c.34G>T)	4	9%
p.Gly12Val (c.35G>T)	13	29%
p.Gly12Ser (c.34G>A)	4	9%
p.Gly12Asp (c.35G>A)	11	25%
Codon 13		
p.Gly13Asp (c.38G>A)	3	6.8%
Codon 61		
p.Gln61His (c.183A>C ; c.183A>T)	3	6.8%
Codon 146		
p.Ala146Pro / p.Ala146Thr / p.Ala146Val (c.436G>C / c.436G>A / c.437C>T)	4	9%
Total	44	100%

Discussion

It has been shown that determination of KRAS mutation status is significant as a tool to predict response of CRC to treatment approaches that target the pathway of EGFR. Currently, the responsible regulatory agencies recommended screening for KRAS mutations on all patients with CRC being contemplated as candidates for anti-EGFR therapy. As the downstream signalling of the EGFR pathway is affected by the activated mutated KRAS, medications used as anti-EGFR requisite the availability of the wild-type gene and protein to be performant. Several techniques are now available for KRAS mutation testing includes numerous PCR-derived and sequencing-based methods. It is vital for this phase of treatment to have approaches that are reliable with higher degree of sensitivity that yield a precise analysis. Many techniques have shown such efficiency, example for that allele-specific PCR techniques and pyrosequencing, and thus afford powerful tests for evaluating FFPE samples. Currently, Sanger sequencing, pyrosequencing, and allele-specific PCR are the more frequently employed analysis techniques.

Herein, we presented our experience in KRAS gene testing using the Idylla™ system (Biocartis, Mechelen, Belgium), one of the most frequently used methods employed to test KRAS mutation status. This technique is in practice since few years in histopathology department at the two KRAS testing centers in Libya that are located in Misrata and Sibrata cities. In general, the instrument is easy to manipulate by the operators with good performance. Performance of this system can be observed through the results shown in table 1 and extracted from reports of 79 cases with CRC.

General limitations of this KRAS testing system

As any other system, analysis of solid tumours for KRAS mutations can be complicated by number of factors such as effect of Formalin fixation on the tissue that can produce alteration of DNA sequence [37, 38]. Furthermore, the testing for KRAS mutations can be challenged by an increased proportion of benign stromal to tumour cells which leads to attenuation of the probably mutant DNA with wild-type KRAS sequences. This can be particularly challenging for post-chemotherapy tumours and tumours with large amount of desmoplasia, where tumour cells can be quite limited in their number. Finally, size of biopsy can be inadequate, diminishing the quantity of DNA obtainable, and many tumours are heterozygous to KRAS, having both mutant and wild-type KRAS, resulting in further dilution of mutant DNA. On those grounds, and for the reason that false-positive or -negative results can lead to crucially insufficient medical care, Analysis of solid tumours for KRAS mutations demands the greatest possible sensitivity and specificity. Moreover, FFPE tissue specimens affected by the rigorous conditions of bone decalcification do not produce integral DNA for molecular analysis.

It important to mention that as specific limitation of this system; The Idylla™ KRAS Mutation Assay allows detection of mutation in three exons named 2, 3, and 4 of the KRAS oncogene. This test performs a group of five allele-specific multiplex PCR reactions, developed for the specific amplification of KRAS gene sequences that are including a mutation in codons 12, 13, 59, 61, 117, or 146 [19]. Thus, this assay can detect twenty-one mutations in KRAS oncogene as the following: seven mutations in exon 2 (codons 12 and 13), nine mutations in exon 3 (codons 59 and 61), and five mutations in exon 4 (codons 117 and 146). Accordingly, the system can provide negative results about mutations in the mentioned condones within its capacity but cannot provide data about mutations in codons not included in its mutation analysis capacity and this result in a false negative result. Also, as a disadvantage, in the event of several mutations are present, just the predominantly identified change, lowest ΔCq value, is given [19].

Conclusion and recommendations

Specific and general limitations related to KRAS testing technique using the Idylla™ system (Biocartis, Mechelen, Belgium) are existing. Accordingly, calibration of the system on regular basis is mandatory. Also, comparing its results with other comparable systems is preferable. Due to the relatively high rates of false-positive or false -negative results of this technique often necessitate involvement of other assays using different approaches to approve the KRAS mutation identified. Moreover, because the status of KRAS mutation alone does not detect all possible cases that respond successfully to anti-EGFR therapy, additional significant genes do have to play a substantial part need to be detected by different systems.

Conclusion

Tamxifen as hormonal therapy in patients with hormonal dependent breast cancer was associated with a longer overall survival. Sweating and hot flashes were the most frequent adverse events. No women experienced serious adverse events such as thromboembolic, cerebrovascular complications and endometrial carcinoma.

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