Original Article

Immunohistochemical assay for detection of K-ras protein expression in metastatic colorectal cancer

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KEYWORDS

K-ras protein; Immunohistochemistry; Metastatic colorectal cancer; EGFR targeted monoclonal antibody

Abstract  Background: The monoclonal antibodies (mAbs) that target the epidermal growth factor receptor (EGFR) had expanded the range of treatment options for metastatic colorectal cancer. However, such type of treatment was shown to be ineffective if there is K-ras mutation. In most previous studies K-ras gene mutation was mainly assessed by PCR.

Aim: Our work is designed to detect K-ras protein expression by immunohistochemistry (IHC) aiming to reach a preliminary method that could be confirmed by PCR and considered an alternative way for the detection of K-ras aberration. We are also aiming to find a relation between K-ras protein expression and K-ras gene mutation.

Materials and methods: Paraffin embedded tissue samples from 26 metastatic colorectal cancer (mCRC) patients were analyzed for K-ras protein expression by IHC using Rap1A polyclonal antibody. Staining patterns were subjectively assessed and correlated with clinicopathological features. The results were statistically evaluated using the Chi-square test.

Results: K-ras cytoplasmic positivity was observed in 42.3% of cases. The positivity was either strong in 26.9% or moderate in 15.4%. With respect to adenocarcinoma variants, 50% of cases were positive for K-ras protein expression while all mucinous and signet ring types were negative. The positivity was noted in 50% of moderately differentiated GII colorectal carcinomas as compared with 38.9% in poorly differentiated GIII. Positive staining was observed in 40% of cases with positive
Introduction

In a cancer pathology registry done by National Cancer Institute, Cairo University, colorectal cancer (CRC) had a relative frequency of 5% of the total estimated cancers and 29% of gastrointestinal malignancies with male predominance ratio of 3:1. According to the study of 427 cases, more than 1/3 of cases were under the age of 45 (early onset) with large tumor size at presentation [1]. Colorectal cancer is a major cause of cancer mortality and morbidity worldwide, representing 8.9% of all cancers [2]. It constituted the third most common cancer among men and women, following breast, lung and prostate. CRC was claimed as the second most common cause of cancer-related death among patients in the United Kingdom and the United States [3].

Despite continuing advances in therapeutic strategies, recurrence rates of CRC are still high, with more prevalence in rectal rather than colon cancer with ratios of 33% and 19%, respectively [4].

Distant metastasis is the main cause of death in CRC patients. Surgical resection remains the only potentially curative option for patients with metastatic CRC. However, curative resection is possible in <25% of patients with stage IV disease [5].

The ras family of proto-oncogenes forms an important subclass of the GTP-binding proteins. The ras protein acts as a signal transducer from membrane receptor (e.g. growth factor receptor) to the nucleus, thus regulates growth and differentiation [6]. The ras gene family encodes similar 21-kD protein (p21ras). Aberrations in ras genes lead to increased and uncontrolled cell proliferation and malignant transformation. The most exhaustively worked ras gene in colorectal cancer is K-ras [7].

On its activation, the K-ras protein is capable of turning off the signaling pathway by catalyzing hydrolysis of guanosine triphosphates to guanosine diphosphates. The most common K-ras mutations (approximately 90%) are found in codons 12 and 13. They are activation mutations, leading to continuous activation of downstream pathways [8].

Mutant, activated forms of K-ras proteins have an impaired intrinsic GTPase activity, which renders the protein resistant to inactivation by regulatory GTPase-activating proteins [9].

Mutations of the K-ras gene have been identified in tissues from both colonic adenoma and carcinoma cases, but at much lower frequencies in adenoma tissues than in carcinoma tissues [10].

K-ras mutation occurs in 30–50% of CRC and has been suggested to be associated with proliferation and decreased apoptosis [11].

Epidermal growth factor (EGF) is part of a complex network of growth factors and receptors that together help to modulate the growth of cells. Many types of cancer have overactive signaling through the epidermal growth factor system [12].

The EGFR controls cell differentiation, proliferation and angiogenesis. It is expressed in 80% of colorectal cancers [13]. EGFR over expression has been suggested as a factor of poor prognosis and associated with a more aggressive clinical progression [14].

Positive expression of EGFR as shown by IHC is not predictive of response to EGFR inhibitors. It is now clear that tumor growth can be driven by the constitutive activation of signaling pathways downstream of the EGFR, such as the RAS-MAPK-P13K pathway regardless of whether the EGFR is activated or blocked [15].

Targeted therapy is considered recently a promising way to cure cancer. K-ras mutation had emerged as an important predictive marker of resistance to anti EGFR monoclonal antibody treatment. However, among colorectal tumors carrying wild-type K-ras, mutation of B-raf or PIK3CA or loss of PTEN expression may be associated with resistance to EGFR-targeted monoclonal antibody treatment [16].

The monoclonal antibodies cetuximab and panitumumab have been developed to target EGFR [17]. Improved response rates and prolonged time to metastasis/survival have been demonstrated with the currently registered EGFR blocking antibodies [18].

The anti-EGFR monoclonal antibodies are approved in the United States for treatment of mCRC refractory to chemotherapy but are not recommended for use in patients with mutations in K-ras codons 12 or 13 [19].

In metastatic colorectal cancer, EGFR instructs the cancer cell to reproduce and metastasize; blocking the EGFR stops this malignant signaling. However, in patients with mutant K-ras, the signaling continues despite EGFR therapy [20].

Determining tumor K-ras status before initiating treatment with an anti-EGFR monoclonal antibody is widely recommended with restriction of such treatment to patients with tumor bearing wild-type K-ras [21]. Therefore, to explore the effect of mutations of K-ras gene on malignant biologic behaviors of rectal cancer and its clinical significance, the mutation patterns of codons 12 and 13 of K-ras gene were detected by PCR [22].

PCR based assays constitute the cornerstone for clinical K-ras testing since these analyses allow high-throughput testing and have a favorable sensitivity, also in samples with low tumor cell content [23].

For investigating the relation between K-ras mutation and its protein ras p21 expression, K-ras codons 12 and 13 point mutations were examined by direct sequence analysis, whereas the ras p21 expression was evaluated using immunohistochemistry [7] and [24].

Immunohistochemistry is widely used to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue.
IHC was used to assess which tumors are likely to respond to therapy, by detecting the presence or elevated level of molecular target [25].

Materials and methods

Paraffin embedded tissue samples from 26 CRC patients with liver metastasis were collected from a private lab and screened for K-ras protein expression by the immunohistochemical method using Rap1A polyclonal antibody. The clinicopathological data including age, sex, microscopic types, histological grade and lymph node status were collected for statistical analysis. All the selected cases were metastatic to reflect the routinely ordered K-ras mutation analysis when EGFR inhibitor therapy was considered.

Immunohistochemistry

Sections (5 μm) were deparaffinized in xylene. Endogenous peroxidase was blocked by 0.3% hydrogen peroxide–methanol for 20 min. After immersing the sections in alcohol they were rehydrated. Antigen retrieval was performed by heating the sections at 100°C for 10 min. After a short rinse in phosphate-buffered saline (PBS), sections were incubated for 30 min with labeled second antibody. PBS washings were followed by incubation for 1 h with a complex of biotinylated horse radish peroxidase and Streptavidin, diluted 1:100 in PBS. Staining was developed in PBS containing 0.05% 3,3-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide. Sections were counterstained with hematoxylin, dehydrated with ethanol, cleared in xylene and mounted with malinol under a cover-slip. The slides were then visualized under a light microscope. Tissue samples to which no primary antibody had been added were used as negative controls.

Evaluation of the result

Cytoplasmic IHC staining of K-ras protein was scored subjectively under light microscope by two observers and the percentage of stained tumor cells (brown color) was expressed depending on previously established criteria of Akkiprik et al. [7] and Sammoud et al. [24] as follows: 3(+) when the majority of cells (>75%) were stained strongly, 2(+) when 25–50% of cells were stained moderately, and 1(+) when the staining was focal (<25%) and weak. Scores 2(+) and 3(+) were considered as expression of K-ras protein while score 1(+) and non-stained cells were considered as negative.

Statistics

The various clinicopathological parameters and K-ras immunostaining results were statistically examined using the Chi-square test for any significant relation.

Results

In this study 26 metastatic CRC cases were included. They had successful immunostaining of the K-ras protein. The age of the patients ranged from 46 to 80 years with a mean age of 63. Among the 26 cases enrolled in this study, 18 (69.2%) cases were males and 8 (30.8%) were females. The male to female ratio was 2.3:1. As regards the microscopic types, 22 (84.6%) cases were adenocarcinoma, 2 (7.7%) were mucinous carcinoma and 2 (7.7%) were signet ring carcinoma. Concerning the histological grading, 8 (30.8%) cases were moderately differentiated GI and 18 (69.2%) were poorly differentiated GIII. All our cases were metastatic Dukes' stage D. Lymph node metastases were positive in 15 (57.7%) cases while free nodal tissues were observed in 11 (42.3%).

IHC results

Among the 26 cases subjected to K-ras immunostaining, 11 (42.3%) cases were positive. The positivity was either strong 3(+) in 7 (26.9%) cases (Fig. 1) or moderate 2(+) in 4 (15.4%) cases (Fig. 2). The remaining 15 (57.7%) cases were negative (3 cases with score 1(+) and 12 unstained cases) (Table 1).}

Clinical correlation

Our study did not reveal any statistical correlation between age and sex and K-ras immunostaining. K-ras positivity was detected in 11 (50%) out of 22 glandular adenocarcinoma cases (7 strong and 4 moderate) while the remaining 11 (50%) cases were negative. The four cases of mucinous (2) and signet ring (2) carcinomas were all negative for K-ras immunostaining. However, the number of cases in signet ring and mucinous types was small and accordingly no statistical difference was calculated (Table 2). Among the 8 (30.8%) cases of moderately differentiated GI adenocarcinoma, 4 (50%) cases were positive (4 strong) and 4 (50%) were negative. Seven (38.9%) out of 18 cases of poorly differentiated adenocarcinoma GIII were found positive (3 strong and 4 moderate) while the remaining 11 (61.1%) cases were negative. There was no significant difference between the two grades in K-ras expression ($p = 0.683$) (Table 3).

In 15 cases with nodal metastasis, K-ras positivity was found in 6 (40%) cases (3 strong and 3 moderate), while the
other 9 (60%) cases were negative. K-ras positive staining was found in 5 (45.5%) cases (4 strong and one moderate) out of 11 nodal free cases while the remaining 6 (54.5%) cases were negative. There was no significant difference between the node status and K-ras expression \((p = 1.000)\) (Table 4).

### Table 1 K-ras immunostaining in metastatic colorectal carcinoma.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>15</td>
</tr>
<tr>
<td>Positive</td>
<td>11</td>
</tr>
<tr>
<td>Strong</td>
<td>7</td>
</tr>
<tr>
<td>Moderate</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
</tr>
</tbody>
</table>

### Table 2 Relation between microscopic types and K-ras immunostaining.

<table>
<thead>
<tr>
<th>Microscopic type</th>
<th>K-ras</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (strong + moderate)</td>
</tr>
<tr>
<td>Glandular adenocarcinoma</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>Signet ring</td>
<td>0</td>
</tr>
<tr>
<td>Number</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Mucinous</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>42.3%</td>
</tr>
</tbody>
</table>

### Table 3 Relation between histological grading and K-ras immunostaining.

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>K-ras</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (strong + moderate)</td>
</tr>
<tr>
<td>Grade II</td>
<td>4</td>
</tr>
<tr>
<td>Number</td>
<td>50%</td>
</tr>
<tr>
<td>Grade III</td>
<td>7</td>
</tr>
<tr>
<td>Number</td>
<td>38.9%</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
</tr>
<tr>
<td>Number</td>
<td>42.3%</td>
</tr>
</tbody>
</table>

### Table 4 Relation between lymph node metastasis and K-ras immunostaining.

<table>
<thead>
<tr>
<th>Lymph node metastasis</th>
<th>K-ras</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (strong + moderate)</td>
</tr>
<tr>
<td>Positive lymph nodes</td>
<td>6</td>
</tr>
<tr>
<td>Number</td>
<td>40.0%</td>
</tr>
<tr>
<td>Negative lymph nodes</td>
<td>5</td>
</tr>
<tr>
<td>Number</td>
<td>45.5%</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
</tr>
<tr>
<td>Number</td>
<td>42.3%</td>
</tr>
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</table>

### Discussion

In comparison with previous researches that aimed to discover K-ras gene mutation using PCR techniques taking into consideration the high cost values and codon specific properties of this method, our work focused on the detection of K-ras protein expression by IHC. Immunohistochemistry was regarded as cost saving, not specific for codons and a preliminary result that could be confirmed by PCR. However, in our work IHC has a limitation in which anti-ras polyclonal antibody can react with both wild and mutant K-ras proteins.

Using Rap1A polyclonal antibody, we detected K-ras protein expression in 42.3% of our cases. Akkiprik et al. [7] revealed expression of ras p21 in 76% of samples tested with Ras Ab-1 polyclonal antibody.

In the present study, we detected K-ras protein expression in 42.3% of cases; this was close to the studies that detected the mutation at codons 12 and 13 of K-ras protein in 39% and 46% [23–26]. However, other studies found the positivity in 36.5%, 32.8%, 23.07% and 11% [27-11-24-7]. Akkiprik et al. [7] reported some drawbacks in the mutation analysis, as mutation sites which are codon specific, have ethnic variations and differences in the dietary components and lifestyle factors.
Comparably high rates of IHC positivity in our study and PCR positivity in other studies may denote sample representation of IHC versus gene mutation. Our results can be used as a nucleus for further investigation to detect K-ras mutation using PCR in the same group of patients aiming to find a relation between the two forms of ras aberrations and disclose an alternative way for detection of K-ras mutation depending on IHC.

Analysis of age and sex of our patients did not reveal any significant correlation with K-ras protein expression. Our results were in agreement with other studies who did not report any relation between age and sex distribution and K-ras gene mutation [7, 11, 27].

In this study K-ras expression was detected in 50% of glandular adenocarcinoma cases. Although all cases of mucinous and signet ring variants were negative for immunostaining, it was not possible to rely on statistical analysis results because of the small number of cases. Liu et al. [11] emphasized that the presence of mucinous differentiation did not make a difference between the wild-type K-ras containing and K-ras mutated CRCs. On the other hand, Akkiprik et al. [7] and Okulczyk et al. [27] found that in the mucus-secreting tumors, mutation incidence detected by PCR methods was significant or close to significant.

In this work K-ras positivity was estimated in 50% of GI moderate to differentiated adenocarcinoma while in GIII poorly differentiated adenocarcinoma the ratio was 38.9%. There was no significant difference between the two histological grades in K-ras expression (p = 0.683).

Our results were in accordance with other results who did not find any significant correlation between K-ras mutation and the histological grades [7-11-26-27].

Our cases were metastatic CRC (Dukes’ stage D). Akkiprik et al. [7] and Okulczyk et al. [27] analyzed Dukes scale against K-ras mutation using PCR methods. They did not note any significant difference between Dukes scale and K-ras mutation. Other studies emphasized that the appearance of distant metastases was not related to K-ras mutations [11–26].

With respect to nodal metastases and their relation to K-ras staining, our results showed positive staining in 40% of cases with nodal involvement vs 45.5% in cases with free nodal status. There was no significant difference between nodal involvement and K-ras staining (p = 1.000). These results were in agreement with Schimanski et al. [26] and Liu et al. [11]; they did not find any significant difference between nodal metastasis and K-ras mutation.

Sammoud et al. [24] showed that the expression of ras p21 was correlated with the advanced age of patients. However, our results were in agreement with Akkiprik et al. [7] who revealed that none of the clinicopathological parameters were related to K-ras protein overexpression. They also emphasized that there was no significant correlation between ras p21 overexpression and K-ras mutations due to the fact that Ras Ab-1 polyclonal antibody used in their IHC analysis detects all over-expressed forms of the protein, including the mutant or wild-type.

Acknowledgement

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References


