ABSENCE OF KRAS MUTATIONS IN CODONS 12, 13 IN COLORECTAL ADENOCARCINOMA FROM NORTHERN PAKISTAN

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ABSTRACT

Background and objectives: KRAS mutations have been studied in various malignancies in many researches conducted on various populations. The objective of this research was to document the KRAS oncogenic mutation pattern particularly in codons 12 and 13 in Northern Pakistani patients of colorectal adenocarcinoma (CRA) presented in Armed Forces Institute of Pathology (AFIP) Rawalpindi.

Place and Duration of Study: Armed Forces Institute of Pathology (AFIP), Rawalpindi. Research was completed in approximately four years from 2011 to 2015.

Methods: It was a cross-sectional study with non-probability convenience sampling performed in a period from January 2010 to April 2015. A total of 181 cases of CRA who underwent intestinal resection were studied. Sections from both tumor and normal intestine for control purpose were prepared. DNA was extracted and PCR was performed with specific primers. Amplified PCR products of 50 samples were analyzed on 1.5% agarose gel. DNA sequencing was done in Centre for Applied Molecular Biology, Lahore.

Results: Among the samples, 75% were males and 25% females with age ranging from 15 to 90 years (mean age 54.68 ± 16.89 years). Higher average age was found to have high tendency to acquire colorectal adenocarcinoma and rectosigmoid was found to be the most important site. Tumors were mostly exophytic, moderately differentiated with majority showing no lymphovascular invasion. No KRAS oncogenic mutation was seen including codons 12 and 13.

Conclusion: The KRAS gene mutations in codon 12 and 13 are either absent in CRA patients of northern area of Pakistan or their frequency may be very low. These cases may be due to mutations of some other genes or because of some non-genetic environmental factors.

Key words: Colorectal adenocarcinoma, KRAS mutations, Northern Pakistan.

INTRODUCTION

Kirsten rat sarcoma viral oncogene homolog (KRAS) is involved in G-protein-mediated signal transduction.1 Mutations in this gene have been seen in many tumors like colorectal cancer, pancreatic cancer, lung cancer and many others. Its activating mutations abolish the intrinsic GTPase activity, resulting in constitutively active KRAS proteins that activate downstream signaling pathways and can lead to carcinogenesis.2 Mutation in KRAS causes lack of response to EGFR-targeted therapy.3 Only patients with wild-type KRAS respond to such drugs.4 KRAS mutations show marked population variations.5,6 A study from central Pakistan showed a relatively very low frequency of mutations of KRAS i.e. 13%.8

The hypothesis was that the frequency and mutational pattern of KRAS oncogene in Northern Pakistani patients presenting with CRC would be different as compared to other populations particularly to the Americans or Europeans, because of different living culture and external environment. If this hypothesis was found to be true, then our oncologists will have to adopt a new policy regarding the use of EGFR drugs in CRA cases.

The objective of this study was to document KRAS mutation pattern in codon 12 and 13 in Northern
Pakistan patients of CRA presented in AFIP Rawalpindi and to correlate the mutated cases with histological variables like age, gender, presenting clinical complaint, tumor site, tumor size, histological differentiation, Duke stage of tumor, nodal metastasis and metastasis in other organs.

**METHODOLOGY**
A total of 181 patients suffering from colorectal adenocarcinoma diagnosed in AFIP from Jan 2010 to Feb 2015 who later on underwent intestinal resection, were included in this study. The age ranged 15 – 90 years averaging 54.68 ± 16.89 years in aggregate and 51.95 ± 17.74 and 55.56 ± 16.52 years for females and males respectively. Tumor tissues of the intestine were compared with their normal intestinal tissues. Detailed pathological and clinical data of these patients were obtained.

Paraffin-embedded blocks were prepared from both tumor and normal intestinal tissues. DNA extraction from both tissues was performed according to instructions of manufacturer (Invitrogen DNA Extraction Kit). Presence of DNA was confirmed under UV illumination. 50 samples for the concerned exon were amplified with sequence specific primers of KRAS. 50mg of genomic DNA was used as a template in 50μl reaction mixture, containing 0.2 units of Taq DNA polymerase, 1.5mM MgCl₂ and 200μm dNTPs. Initial denaturation was carried out for 5 minutes at 95°C, then 35 cycles, each of denaturation at 95°C for 45 seconds, annealing for 45 seconds at annealing temperature, followed by extension at 72°C for 45 sec. Final extension at 72°C for 10 min. 10μl of amplified PCR products were checked on 1.5% agarose gel.

Amplified DNA was subjected to electrophoresis on 1.5% Agarose gel and then stained by Ethidium Bromide for 30 min. Gene sequencing of purified DNA was performed in Centre for Applied Molecular Biology (CAMB), Lahore. ABI Big Dye version 3.1 sequencing kit was used for sequencing PCR with some modifications in the manufacturer’s provided sequencing protocol. Purified sequencing PCR products were electrophoresed on ABI 3130XL and ABI 3730 genetic analyzers using 36cm capillary and POP-7 polymer.

**KRAS primers with respective annealing temperatures.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1 F primer:</td>
<td>ttaaccttatgtgtgacatgttetaa</td>
<td>60°C</td>
</tr>
<tr>
<td>Exon 1 R primer:</td>
<td>agaatggctgctgacgactaa</td>
<td></td>
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<tr>
<td>Exon 2 F primer:</td>
<td>tgcatggcattgagcaaaagc</td>
<td>60°C</td>
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<tr>
<td>Exon 2 R primer:</td>
<td>ecagaectgttctctctcctc</td>
<td></td>
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<tr>
<td>Exon 3 F primer:</td>
<td>tggacaggttgggttaaagaga</td>
<td>56.9°C</td>
</tr>
<tr>
<td>Exon 3 R primer:</td>
<td>cctgtctctctctctcct</td>
<td></td>
</tr>
<tr>
<td>Exon 4 F primer:</td>
<td>caactgaacactgtaagaag</td>
<td>60°C</td>
</tr>
<tr>
<td>Exon 4 R primer:</td>
<td>gacctggtctctctctctctg</td>
<td></td>
</tr>
</tbody>
</table>

Data was analyzed using ABI Sequencing Analysis software version 5.2.

**RESULTS**
Large majority of patients were either residents or were residing in Khyber Pakhtunkhwa (KPK) since many years. 75 percent males and 25 percent females presented with CRA with age ranging from 15 to 90 years. Samples in this study were divided into three age groups, less than 40 years of age, between 40 to 59 years and more than 60 years. Our results indicated that Northern Pakistani CRA patients, both males and females, with higher average age had high tendency to acquire this malignancy. The risk factor to get CRA was higher in the third age group. In total of 181 cases, 36 i.e. 20 percent were found to be more than 40 years of age and 53 i.e. 29 percent were found to be within 40 to 59 years and more than 60 years. Our results indicated that Northern Pakistani CRA patients, both males and females, with higher average age had high tendency to acquire this malignancy. The risk factor to get CRA was higher in the third age group. In total of 181 cases, 36 i.e. 20 percent were found to be more than 40 years of age and 53 i.e. 29 percent were found to be within 40 to 59 years and more than 60 years. In the last group, 92 i.e. 51 percent were found to be involved with CRA.

According to the site of tumor or anatomical part involved, all CRA tumors were divided into specified sections of colon. Tumors of ascending colon and cecum were grouped together. They were 47 in number.
i.e. 26 percent. Tumors of descending colon were less in number i.e. ten with a percentage of five. Tumors of rectum, rectosigmoid junction and sigmoid were grouped together. They were 97 in total i.e. 54 four percent. The most common location of CRA in our study group was rectum i.e. 45. This location was followed by Sigmoid, 28and then by the ascending colon 26. The least common site involved was hepatic flexure where the number of CRA was only three. Splenic flexure and transverse colon tumors were six and 11 respectively. Grossly 96 i.e. 53 percent were exophytic adenocarcinomas. They were causing colonic obstruction due to a protruding growth. 85 i.e. 47 percent tumors were found to be infiltrative in nature causing stricture formation. Out of the total, 85 adenocarcinomas were moderately differentiated while 78 showed well differentiation. Fifteen cases were poorly differentiated. Only three cases were quite undifferentiated. 64 percent CRA were without any lymphovascular invasion. TNM classification method was followed to classify the tumors. Tumor stage was available for 143 patients included in the study. 117 patients were found to be in T3 stage followed by 14 patients in T4, eight in T2 and four in T1 stage. None of our cases showed any KRAS oncogenic mutation including codons 12 and 13.

A common genetic mutation in exon 1 at codon 12, glycine into serine transition (G>A) would be expected. This mutation in exon 1 has not been detected in our CRA patient. The figure shows normal sequence pattern in one of our sample.

**DISCUSSION**

The gene-environmental interaction can influence the molecular characteristics of CRA in different populations belonging to different ethnicity and environmental exposures. In our study on CRA, males were the predominant group who presented with this malignancy. Male predominance has been reported in some studies but this sex distribution was found to be different with respect to most other studies. This finding may have many social reasons as this community is quite restricted and people are not so much educated. Moreover, the number of our patients was not enough to derive a conclusive comment that males of northern areas have more chances of this malignancy. Certainly this needs a much wider scale epidemiological study of this region because there are many social constraints prevalent in this society. Majority of our patients showed tendency to get CRA in the older age. The frequency of rectosigmoid tumor was found to be higher i.e. 55 percent. There is quite a difference in the grading of CRA in different studies. In our study, most of the CRAs were moderately differentiated. Similarly, in cohort study of Netherlands the tumors were mostly moderately differentiated. The difference between well and moderately differentiated tumors is often ambiguous because of subjective reasons.

Both genetics and environmental triggers, particularly diet composition, play an important role in pathogenesis of many common tumors like colorectal adenocarcinoma. Mutational spectrum of KRAS in CRA has been widely studied in Western countries as compared to the Asian world. Even within Western researches, much discrepancy has been seen in the incidence, frequency and other aspects of CRA. As per the published data, only one study of KRAS has been carried out in central Pakistan which has shown relatively very low frequency of mutations in colorectal malignancy. Our study of CRA patients from northern areas of Pakistan has not shown any mutation of KRAS. Although we know that people of this area have a different genetics and environment but we were expecting a frequency of KRAS mutations in these people close to the frequencies observed in the neighboring countries like Iran. Surprisingly the result was different.

We tried to critically analyze all the reasons for this negative result. Out of these, the most important is the sensitivity and specificity of mutation detection method. In this study, method of direct sequencing of purified PCR fragments was used which is considered to be the most highly sensitive and specific detection method to analyze KRAS mutations. This method is well known to identify mutations in DNA samples which contain at least 5% or more mutated DNA. Moreover we did gene sequencing in the well renowned institute of Pakistan, Centre of Applied Molecular Biology Lahore.

We were quite aware of the fact that due to limited resources we had to select lower number of the patients. Certainly this may be a very valid reason of missing the mutation as has been highlighted by various studies. Financial constraints forced us to limit our number of patients. Anyhow, even if we would have got extra finances to increase the number of our cases, we do not expect a high percentage of KRAS mutations in light of these results. Another possibility of missing the mutation may be due to a general impression that processing of tumor tissues through paraffin-embedding process can affect the DNA. However this reason is not justified to this much extent. In one study the KRAS status as determined in paraffin-embedded tissue was also found to be true in fresh tumor tissue in nine out of ten specimens. Only one specimen which showed mutation in paraffin-embedded block did not show the mutation in fresh tumor tissue. This may be related to heterogeneity in the tumor or a lack in reproducibility of that sample. Moreover, a lot of studies from different countries have used these tissue blocks. This indicates that tissue processing through paraffin does not have a significant effect on reliability of mutational analysis.

There is a possibility that may be some particular environmental factors or other gene mutations like APC, p53, DCC etc. might be playing their role in caus-
sation of CRA in northern area patients. Due to these different genetic and environmental factors in various countries, a wide frequency range of \(KRAS\) mutations have been reported even in benign condition.\(^{15-17}\) In colorectal malignancy, the incidence of \(KRAS\) mutations has a very wide range in different studies.\(^{18-22}\) It is quite clear that \(KRAS\) mutations may show much variation not only in CRA cases but also in various other malignancies e.g. pancreatic carcinoma, a well-known carcinoma for \(KRAS\) mutations. In the study of Ming et al., 2000,\(^{22}\) frequency of \(KRAS\) mutations in pancreatic carcinomain Chinese patients was found to be significantly lower than that in the Japanese. Even within developed countries, geographical differences in \(KRAS\) mutation pattern have been reported.\(^{23,24}\) Not only the ethnic and racial factors are important but also the life-styles may be closely associated with status of cancer-related gene.\(^{22}\) Involvement of some specific environmental factor is further strengthened by finding of novel mutations of \(KRAS\) in some populations.\(^{25}\) There can be some other non-genetic factors which have already been found to modulate the risk of CRA including lack of exercise, obesity, high fatty diet, alcohol, tobacco etc. It is suggested that a much wider scale study should be conducted with collaboration of pathologists, molecular biology experts and the experts from community health department.

It is concluded that majority of the northern area patients has tendency to get CRA in the older age. Mostly the tumors are exophytic and moderately differentiated. Rectosigmoid is a favored location. \(KRAS\) gene mutations, particularly in codons 12 and 13 are either absent or their frequency may be very low. These CRA cases may be due to mutations of some other genes or because of some non-genetic environmental factors.

**Contribution of Authors**

AQ: The main researcher who wrote, planned and performed the whole research. AH Nagi: The supervisor of the research work. BMTK: Co-Supervisor the research work at AFIP, Rawalpindi. MGAKN: Co supervisor of research work conducted in Army Medical College, Rawalpindi. SJ: Co-Supervisor of research in UHS, Lahore. AM: Co-Supervisor of research in Army Medical College Rawalpindi. FS: Co-Supervisor of research in CEMB, Lahore. SR: Provided all technical support for research.

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**REFERENCES**

18. Hughes R, Cross AJ, Pollock JR and Bingham S. Dose-


