Spectrum of \textit{K ras} mutations in Pakistani colorectal cancer patients

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Abstract

The incidence of colorectal cancer (CRC) is increasing daily worldwide. Although different aspects of CRC have been studied in other parts of the world, relatively little or almost no information is available in Pakistan about different aspects of this disease at the molecular level. The present study was aimed at determining the frequency and prevalence of \textit{K ras} gene mutations in Pakistani CRC patients. Tissue and blood samples of 150 CRC patients (64\% male and 36\% female) were used for PCR amplification of \textit{K ras} and detection of mutations by denaturing gradient gel electrophoresis, restriction fragment length polymorphism analysis, and nucleotide sequencing. The \textit{K ras} mutation frequency was found to be 13\%, and the most prevalent mutations were found at codons 12 and 13. A novel mutation was also found at codon 31. The dominant mutation observed was a G to A transition. Female patients were more susceptible to \textit{K ras} mutations, and these mutations were predominant in patients with a nonmetastatic stage of CRC. No significant differences in the prevalence of \textit{K ras} mutations were observed for patient age, gender, or tumor type. It can be inferred from this study that Pakistani CRC patients have a lower frequency of \textit{K ras} mutations compared to those observed in other parts of the world, and that \textit{K ras} mutations seemed to be significantly associated with female patients.

Key words: Colorectal cancer; Kirsten rat sarcoma viral oncogene homologue; Mutation analysis; Risk factors for CRC; Cetuximab therapy

Introduction

Kirsten rat sarcoma viral oncogene homologue (\textit{K ras}) is one of the ras family proteins that hydrolyze GTP, and it plays an essential role in several signalling pathways that regulate normal cellular proliferation by interacting with other regulators and effectors. Mutations in this gene are considered to be an essential step in the initiation of many cancers and the maintenance of malignant phenotypes (1). These mutations have been reported in colorectal cancer (CRC; 25-45\%), pancreatic cancer (95\%), thyroid cancer (55\%), lung cancer (35\%), and breast cancer (5-10\%) (2).

The incidence of CRC is increasing daily worldwide. It has relatively low incidence in Asia and Africa, but is high in western countries, including Northern Europe, New Zealand, and Australia. In Pakistan, it is the seventh most common cancer in women and the ninth most common cancer in men (3). Mutations in \textit{K ras} are considered to be the key step in CRC tumorgenesis, and codons 12, 13, and 61 are considered hot spots for mutations. Different environmental factors such as diet-related carcinogens (polycyclic aromatic hydrocarbons) could induce specific mutations in \textit{K ras} (2,4). \textit{K ras} is a proto-oncogene under normal physiological conditions. It has a dual function, playing an important role in carcinogenesis as well as in inhibition of cancer development. When mutated, \textit{K ras} changes into an oncogene. The wild-type \textit{K ras} behaves as an anti-oncogene and could step down the growth and cell cycle of colon carcinoma cells (5).

\textit{K ras} mutational status has a considerable impact on the selection of anticancer therapy for CRC patients. Tumors harboring \textit{K ras} mutations will not benefit from epidermal growth factor receptor (EGFR)-targeted therapies. These mutations would, therefore, negatively predict the success of anti-EGFR therapies. In the present study, the status of \textit{K ras} mutations in Pakistani CRC patients has been analyzed by denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP) analysis, and nucleotide sequencing. The status of mutations has also been correlated with various clinical pathological characteristics of the patients.

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Received April 7, 2013. Accepted July 29, 2013. First published online November 29, 2013.

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Patients and Methods

The study was approved by the Ethics Committee of School of Biological Sciences, Lahore, the Advanced Board of Studies and Research of University of the Punjab, Lahore, and the Internal Review Board, Shaukat Khanum Memorial Cancer Hospital and Research Centre, Lahore, Pakistan.

Patients

A total of 150 CRC patients were enrolled with written informed consent during the years 2007 to 2010 from Shaukat Khanum Cancer Hospital and Research Centre, Services Hospital, and Jinnah Hospital (all in Lahore, Pakistan). Patients were interviewed, and complete information about age, gender, nationality, lifestyle, economic condition, dietary habits, family history, smoking habits, presence of any type of addiction, presence of any type of tumor, and other health problems were recorded. A piece of colorectal tumor tissue and its adjacent normal tissue, about 12 cm away from the tumor location, were excised by the surgeon and immediately snap frozen in liquid nitrogen. Blood samples (3-5 mL) of the patients were drawn, and, in addition, paraffin-embedded tissue samples of study subjects were used for analyses. Genomic DNA was extracted from the blood samples following the protocol of Helms (6), while genomic DNA from freshly frozen tissues and paraffin-embedded tissue samples was extracted using a Puregene DNA extraction kit.

DGGE

For the detection of mutations, a full coding region of K ras, with all the intron-exon boundaries, was analyzed using DGGE, according to the protocol of Hayes et al. (7). Two sets of primers (Oligo™, Macrogen, Korea), external and internal, were used for nested PCR, and a GC-rich fragment (GC clamp) was added at the 5'-end of one of the primers in each set of internal primers.

The first round of PCR was carried out using 50 ng genomic DNA as template, 0.2 U Taq DNA polymerase (Fermentas Life Sciences, USA), 2.5 mm dNTPs, and 20 pmol of each external primer. Annealing was carried out for 60 s at the specific annealing temperature of each exon (54°C for exons 1, 2, and 4, and 52°C for exon 3). In the second round of PCR, 1 μL of the respective external amplified product was taken as template in a 5-μL reaction mixture, containing 0.2 U of Taq DNA polymerase (Fermentas Life Sciences), 2.5 mm dNTPs, and 20 pmol of each internal primer. Annealing was carried out for 60 s at 55°C.

Amplicons were electrophoresed on 9% polyacrylamide gel (acrylamide:bis, 37.5:1) with 20-60% urea/formamide on a DCode mutation detection system (Model 475; Bio-Rad, USA). Gels were run parallel to the direction of electrophoresis at 120 V and 59°C. The stained gels were carefully analyzed, and the samples showing any shift in mobility were further processed for nucleotide sequencing.

RFLP analysis

Hotspot codons (codons 12, 13, and 61) of K ras were also analyzed by RFLP. A single nucleotide mismatch at the 3'-end of primers was created by mutagenic PCR to produce a BstNI or MvaI enzyme (ER# 0551; Fermentas Life Sciences) recognition sequence at exon 12 (8). This cleavage site would be absent in mutated exon 12. For restriction analysis, 20 μL PCR product (250 ng DNA) was digested with 20 U BstNI and incubated overnight at 37°C. All the restricted samples were checked on 9% acrylamide:bis acrylamide gel. In the case of the wild-type allele, BstNI digestion of exon 12 (exon 1) would result in two bands of 29 and 128 bp, whereas the mutant would show an uncut product of 157 bp.

Mutations at codon 13 were analyzed by following a protocol of Hatzaki et al. (9), with some modifications. An HaeIII recognition sequence was introduced in the PCR-amplified wild-type alleles by mutagenic PCR. For restriction analysis, 20 μL PCR product (250 ng DNA) was digested with 20 U HaeIII or BsuRI (ER# 0151; Fermentas, Life Sciences) overnight at 37°C. On HaeIII digestion, wild-type codon 13 resulted in three bands (additional fragment due to an internal HaeIII recognition site) of 85, 48, and 26 bp, but mutant allele was digested into two bands of 85 and 74 bp.

Codon 61 was analyzed following the protocol of Sills et al. (10). For this purpose, XbaI (Fermentas Life Sciences, #ER0681), MseI or TruI (Fermentas Life Sciences, ER# 09825), and TaqI (Fermentas Life Sciences, ER# 0671) enzymes were used. Wild-type codon 61 was not cut by these enzymes. All the restricted samples were checked on 9% acrylamide:bis acrylamide gel. The gel was run at 100 V for 1.5 h at room temperature and stained with ethidium bromide and visualized under a UV transilluminator.

Nucleotide sequencing

The presence of mutations was finally confirmed by DNA sequencing. Suspected samples were purified using a QIA quick gel extraction kit (cat.#28704, Qiagen, Germany) according to the manufacturer's instructions and sequenced by capillary electrophoresis-based sequencing services (ABI; 3730xl DNA Analyzer; Applied Biosystems, Singapore).

Classification of CRC tumors

Staging of tumor samples. For staging of tumor samples, a tumor-node-metastasis (TNM) classification system was followed (11). According to this system, five stages of CRC tumors have been recognized: T0, no evidence of cancer in the colon or rectum; T1, tumor has grown into the submucosa, but no penetration through...
muscularis propria; T2, tumor has invaded the muscularis propria (a deeper, thick layer of muscle that contracts to force the contents of the intestines along); T3, tumor has grown through the muscularis propria and into the subserosa (a thin layer of connective tissue beneath the outer layer of some parts of the large intestine) or into tissues surrounding the colon or rectum; T4, tumor has invaded other organs or has caused a perforation (hole) in the wall of the colon or rectum.

Nodal stage. The following classification was used for nodal stage information: N0, no regional lymph node metastasis (the cancer has not spread into the regional lymph nodes); N1, metastatic involvement in 1 to 3 regional lymph nodes; N2, metastatic involvement in 4 or more regional lymph nodes.

Metastatic stage. The following classification was used for metastatic stage information: M0, absence of distant metastasis; M1, presence of distant metastasis.

Statistical analysis
Age and gender of patients, location of the tumor, histological differentiation, and presence of mutation were compared and analyzed by chi-square through contingency table tests. Data are reported to be significant when P was less than 0.05.

Results and Discussion
The spectrum of \( K \text{ ras} \) mutation in different cancers has been studied in western populations, but comparatively little information is available for developing countries. Thus, for studying and comparing the molecular characteristics of CRC and analysis of related genes from populations having different ethnicity and environmental exposures, it is necessary to understand the gene-environment interaction. In addition, about 98% of CRC having \( K \text{ ras} \) mutations show resistance to Cetuximab or Erbitex, the drug currently used for treatment of CRC. More than 10 anti-\( K \text{ ras} \) chemical agents (\( K \text{ ras} \) enzyme inhibitors) are under clinical trials. Some of these are showing good results and ultimately may prove to be effective treatments for some tumor types. The analysis of \( K \text{ ras} \) can therefore be useful in the customizing or selection of adjuvant therapy.

It was observed that, in Pakistan, CRC was more prevalent in males than in females. Of a total of 150 patients, 64% were male and 36% were female CRC patients (\( P<0.05 \)). The tendency to develop CRC was higher in older age groups (\( \geq 40 \)) for both genders, i.e., 65% of patients were \( \geq 40 \) years of age and 35% were \(<40 \) years of age. Categorization of tumor types showed that 69% of the total tumors were in the T3 stage, followed by 19% in T4, 7% in T1, and 5% in T2 (Figure 1). Data on nodal stage information, which was available for all patients except for five (2 females and 3 males) showed that 56% of the total patients had N2, 26% had N1, and 18% had N0 nodal stage tumor. There were 67% patients without metastasis, whereas 69% of the total tumors were found to be in the T3 stage followed by 19% in T4, 7% in T2, 5% in T1, and 1% in T0.

| Parameters          | Cases with mutant \( K \text{ ras} \) (n=20) | Cases with wild-type \( K \text{ ras} \) (n=130) |
|---------------------|---------------------------------------------|-----------------------------------------------|
| Age (years)         |                                             |                                               |
| \(<40\)             | 5                                           | 47                                            |
| \(\geq 40\)         | 15                                          | 83                                            |
| Gender              |                                             |                                               |
| Male                | 9                                           | 89*                                           |
| Female              | 11                                          | 43                                            |
| Location of tumors  |                                             |                                               |
| Colon               | 5                                           | 23                                            |
| Rectum              | 11                                          | 67                                            |
| Sigmoidal           | 3                                           | 21                                            |
| Rectosigmoidal      | 1                                           | 19                                            |
| TNM stage           |                                             |                                               |
| T1                  | 1                                           | 6                                             |
| T2                  | 1                                           | 10                                            |
| T3                  | 14                                          | 89                                            |
| T4                  | 4                                           | 25                                            |
| N0                  | 1                                           | 25                                            |
| N1                  | 5                                           | 33                                            |
| N2                  | 14                                          | 67                                            |
| M0                  | 16                                          | 80*                                           |
| M1                  | 4                                           | 44                                            |
| Tumor differentiation|                                             |                                               |
| Poor                | 8                                           | 50                                            |
| Moderate            | 5                                           | 35                                            |
| Well                | 7                                           | 45                                            |

See Material and Methods for explanation of tumor-node-metastasis (TNM) staging. *\( P<0.05 \), Student t-test.
T1, and 5% in T2. Of a total of 150 studied tumors, 39% (58/150) were poorly, 27% (40/150) were moderately, and 35% (52/150) were well-differentiated tumors (Table 1).

In the present study, the observed frequency of \( K \) ras mutation was 13%. Of a total of 150 samples studied, 20 tumors were mutated (Figure 2). Comparison of the specific types of \( K \) ras mutations reported from other parts of the world revealed that Pakistanis had a lower rate of \( K \) ras mutations compared with others. According to our information, there have not been any data published about CRC in the Pakistani population until now. The reported rate of CRC varies among different populations, and it has been observed to be associated with different environmental factors, particularly diet composition. The overall \( K \) ras mutation rate in CRC was 37% in the Iranian population (with prevalent mutations at codons 12 and 13) (3), 46% in the Italian population (with prevalent mutations at codon 13) (2), 23% in Indian Kashmir (3), and 35% in China (12).

### Specific patterns of mutations in \( K \) ras in Pakistani CRC patients

Sequencing was done for samples showing a shift in mobility on DGGE or mutation by RFLP. Most of the tumor samples showed heterogeneity in \( K \) ras. Codon 12 was found to be the major culprit of the event by contributing 60% (12/20) of total mutations, followed by codon 13 with 35% (7/20), and codon 31 with 5% (1/20) mutations. There was no mutation at codon 61 of \( K \) ras in the Pakistani population (Table 2). The dominant mutation at codon 12 was a G to A transition (in the second base codon) in 8 of 12 codon 12 mutants (67%), substituting glycine (GGC) with aspartic acid (GAT). It was followed by a G to T transversion (in the second base codon) in 3 of

| I.D. | Age (years) | Gender | CRC type | TNM staging | Tumor differentiation | Codon | Mutation identified |
|------|-------------|--------|----------|-------------|----------------------|-------|---------------------|
| T02  | 30          | Male   | Rectal   | T4N2M1      | Well                 | 13    | GGC to GAC          |
| T04  | 30          | Female | Rectal   | T3N1M0      | Poor                 | 12    | GGT to GTT          |
| T17  | 45          | Male   | Colonal  | T2N2M0      | Moderate             | 31    | GAA to AAA          |
| T21  | 60          | Female | Colonal  | T3N2M0      | Moderate             | 12    | GGT to GTT          |
| T22  | 68          | Male   | Colonal  | T3N0M0      | Poor                 | 12    | GGT to GTT          |
| T25  | 55          | Female | Rectal   | T3N2M0      | Poor                 | 12    | GGT to GTT          |
| T36  | 23          | Female | Rectal   | T3N1M0      | Well                 | 13    | GGC to GAC          |
| T40  | 45          | Male   | Rectal   | T3N2M0      | Well                 | 13    | GGT to GTT          |
| T41  | 46          | Male   | Rectal   | T3N2M0      | Poor                 | 12    | GGT to GTT          |
| T51  | 22          | Male   | Rectal   | T4N2M0      | Poor                 | 12    | GGT to GTG          |
| T53  | 18          | Female | Rectal   | T3N2M0      | Poor                 | 12    | GGT to GTG          |
| T65  | 50          | Male   | Rectal   | T3N1M0      | Well                 | 12    | GGT to GTG          |
| T72  | 61          | Female | Sigmoidal| T3N2M0      | Poor                 | 12    | GGT to GTT          |
| T81  | 51          | Female | Rectal   | T4N2M0      | Poor                 | 12    | GGT to GTT          |
| T92  | 42          | Male   | Colonal  | T4N2M1      | Well                 | 12    | GGT to GTG          |
| T94  | 42          | Male   | Rectal   | T3N2M0      | Moderate             | 13    | GGC to GAC          |
| T105 | 50          | Female | Sigmoidal| T3N2M0      | Moderate             | 12    | GGT to GTT          |
| T128 | 51          | Female | Sigmoidal| T3N2M1      | Well                 | 13    | GGC to GAC          |
| T139 | 47          | Female | Colonal  | T1N1M1      | Well                 | 13    | GGC to GAC          |
| T147 | 52          | Female | Rectosigmoidal | T3N1M0 | Poor | 13 | GGC to GAC          |

See Material and Methods for explanation of tumor-node-metastasis (TNM) staging.

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**Figure 2.** Restriction fragment length polymorphism on 12% polyacrylamide gels (acrylamide:bis-acrylamide, 30:0.8) for codon 12. Sample: lane 1, healthy control, unrestricted (157 bp); lane 2, wild-type control (128 and 29 bp); lane 3, heterozygous mutant (157, 128, and 29 bp); lanes 4 to 7, wild-type \( K \) ras (128 and 29 bp); lane M, DNA marker (100 bp, Fermentas).
12 codon 12 mutants (25%), substituting glycine (GGC) with valine (GTT), and a G to T transversion (in the first base codon) in 1 of 12 codon 12 mutants (8%), substituting glycine (GGC) with cysteine (TGT).

All codon 13 mutants had a G to A transition at the second base of the codon, substituting glycine (GGG) with aspartic acid (GAC) (Figure 3). The crystal structures of the K ras protein with substitutions of glycine with aspartic acid or valine at codon 12 have been compared, and it was proposed that the mutant ras with a glycine-to-valine change at codon 12 may generate a more stable signal compared to the glycine-to-aspartic acid mutant or wild-type ras protein (13). A novel heterozygous mutation, i.e., GAA (glutamic acid) to AAA (lysine), was found at codon 31 in one of the tumor samples. This mutation was, however, absent in normal tissue and blood of the respective subjects. Data have already been published by Murtaza et al. (14).

Mutations at codons 12 and 13 of the K ras gene were also predominant in CRC in the Iranian population. The frequency of this gene mutation could be similar to other populations, but the mutational spectrum could be influenced by environmental and genetic factors (3). For example, the major ingredients of the Italian diet are pasta and refined grains (15), while the Pakistani diet is wheat-based with a variety of unrefined whole grains, which may reduce the risk of several types of cancers, particularly of the digestive tract (16).

Some of the N-nitroso compounds, produced during the processing of red and processed meat, could induce G to A transitions (17), possibly due to the formation of guanine adducts in the DNA and the silencing of the DNA repair protein (O6-methylguanine DNA methyltransferase) (18). Promoter hypermethylation of this particular gene, which leads to its silencing, has often been observed in CRC (19). G to T transversions are considered to be induced by aromatic hydrocarbons present in dietary components (14), smoked and barbecued meat (20), and cigarette smoke (21).

The biological relevance for codon 13 mutations to Dukes’ stage has been reported (22), and an association between specific K ras mutations and advanced stages of CRC has previously been reported in some laboratories (23). Dietary factors may also modify the growth of tumors harboring specific K ras mutations (24). For example, high consumption of refined grains is a dietary pattern directly associated with increased CRC risk. It has been reported that diet-related carcinogens, such as heterocyclic amines from heavily cooked meat, may induce K ras mutations and that the intake of fruits, vegetables, or antioxidants lowers the risk of CRC (25). Besides that, less physical activity (26), alcoholism (27), air pollution (28), and smoking (29) have also been proven to be possible risk factors for CRC.

For establishing the genotype/phenotype correlation, the association between K ras mutational status and clinical-pathological characteristics (age, gender, tumor stage, grade, etc.) was studied. There was only a significant association of K ras mutational status to gender and metastatic state. No significant differences in the prevalence of K ras mutations were observed for patient age, gender, and tumor type. Of a total of 54 female subjects included in the study, 11 (20%) showed mutations in K ras, whereas of a total of 96 male subjects, only 9 (9%) harbored K ras mutations, which is in concordance with some recent studies (12). These mutations were found predominantly in tumors without metastasis. Only 20% of the mutant tumors had the distant metastatic stage. It has been observed that almost 37% of metastatic CRC cases have been found to harbor K ras mutations, and none of them showed a response to Cetuximab, a chemotherapy currently being used for CRC treatment (30).

A positive association between K ras mutational status and the advanced stages of tumor has recently been reported by Naguib et al. (31). In the present study, K ras was found to be mutated in 18% of the total colonic tumors, 13% of the total rectal tumors, 13% of the total sigmoidal tumors, and 10% of the total rectosigmoidal tumors.

Comparison of our data to those of others suggests that K ras mutations can be differentially influenced by genetic and environmental factors. The data acquired by our study were based on in vitro experimentation, which was conducted on a smaller number of patients. For obtaining comprehensive population-based data about the prevalence of CRC, the number of patients to be registered must be increased and laser microdissection.
should be used to collect the tumor samples. We conclude that the incidence of CRC among Pakistani patients is higher in men compared to women. The rate of K\textsuperscript{ras} mutations in Pakistani CRC patients is low compared to that of other regional countries. Furthermore, the mutations at codons 12 and 13 are most prevalent. Female patients are more susceptible to acquiring K\textsuperscript{ras} mutations, and colonic tumors have a higher susceptibility to harbor these mutations. These mutations are predominant in patients with a nonmetastatic stage of CRC.

Acknowledgments

We are greatly indebted to Dr. Gayatri Ramakrishna, Center for DNA Fingerprinting and Diagnostics, Hyderabad, India, and Dr. Farrukh Kamal, Jinnah Hospital, Lahore, Pakistan, for their valuable suggestions and cooperation.

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