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Identification of a Novel Mutation in Codon 31 of *Kirstein Rat Sarcoma Viral Oncogene Homologue* in Colon Cancer: Another Evidence of Non-Canonical Mutational Pathway

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Abstract.- Colorectal cancer (CRC) is a disease of the large intestine and regarded as a multistep process resulting from accumulation of different genetic alterations like activation of proto-oncogenes and inactivation of tumour suppressor genes. *Kirstein rat sarcoma viral oncogene homologue (K ras)* is a proto-oncogene and mutations in this gene are considered to be involved in the early transition from normal colonic epithelium to premalignant tissue. Mutations at codon 12, 13, and 61 are widely studied and considered to be responsible to account for most Ras-mediated carcinogenesis but some of the recent evidences have suggested that non canonical mutations (outside of codons 12, 13, and 61) may also contribute to the genetic aberrations leading to the Ras-associated oncogenesis. We have analyzed the whole coding region of the gene and report a non-canonical novel heterozyogous mutation at codon 31 in a colonic tumor. Polymerase Chain Reaction (PCR)-Denaturing Gradient Gel Electrophoresis (DGGE), subsequent sequencing data revealed, G to A transition at first base of codon substituting glutamic acid (GAA) to lysine (AAA). Since this mutation has never been reported before in CRC, it is a novel variant in *K ras.*

Key words: colorectal cancer, Kirstein ras sarcoma viral oncogene homologue, mutation analysis.

INTRODUCTION

The incidence and prevalence of colorectal carcinoma (CRC) varies from country to country, particularly, depending on different genetic and environmental factors present in that particular area. Its highest incidence has been reported in North America, Europe and lowest in Asia, Africa and South America. An estimated, one million new cases are being diagnosed annually and approximately half of them die (Karimi *et al*., 2011). It is the 9th common cancer in men and 7th in women in Pakistan (WHO; Globocan, 2008).

CRC is considered to be a multistep process. Early genetic alterations in the epithelial cells results in the mutational alterations and production of malformed proteins, which are absent or can no longer function normally (Yeatman, 2001). Oncogenic mutations in Kirsten rat sarcoma viral oncogene homologue (*K ras*) is considered to be one of the early genetic aberrations which is consistently associated with stepwise progression of colorectal neoplasms. It is one of the ras family proteins that hydrolyze GTP and performs an essential function in normal tissue signaling by interacting with other regulators and effectors. Gain-of-function mutations in this gene are considered to be an essential step in EGFR independent activation of intracellular signaling pathways, resulting in tumor cell proliferation, protection against apoptosis, increased invasion and metastasis and progression of many cancers (Kranenburg, 2005).

K ras mutations have been reported in CRC (25-45%), pancreatic cancer (95%), thyroid cancer (55%), lung cancer (35%) and breast cancer (5-10%) (Bishehsari *et al.*, 2006). In CRC the presence of *K ras* mutations are considered to be associated with diet related carcinogens, such as heterocyclic amines and other polycyclic aromatic hydrocarbons (Tachino *et al*., 1995). The inhibitory effects of wild

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type *K ras* on colorectal tumor growth and proliferation have been reported (Li *et al.*, 2003). Wild type *K ras* could down regulate the growth and cell cycle of colon carcinoma cells by significantly increasing the cells at G0-G1 stage and decreasing the cells at stage G2-M (Li *et al.*, 2003: Hong *e al.*, 2007). These findings suggest that interphase cells are inhibited to advance into proliferation (Hong *et al.*, 2007). *K ras* mutational status may have a considerable impact on therapeutic decisions for CRC patients. Downstream signaling activities in tumors having *K ras* mutations will not be ablated by blocking EGFR at the receptor level (Khambata-Ford *et al*., 2007) and patients with mutant tumors will not benefit from EGFR-targeted therapies. Cetuximab and panitumumab (anti-EGFR antibodies) are currently being used for the treatment of CRC patients but only a fraction of these patients can achieve a clinical response with these targeted therapies.

These mutations confer resistance to these drugs and result in reduced prediction of progression free and shorter overall survival (Amado *et al*., 2008; Lievre *et al*., 2008), thus *K ras* mutational status negatively predicts the success of anti-EGFR therapies. For the treatment of CRC, different novel therapeutic interventions are currently under clinical trials which include drugs to inhibit blood vessel formation and drugs to target specific abnormal proteins such as Ras (De-Roock *et al*., 2008). There are no validated testing methods for the detection of these mutations. Most of the commercial test kits available in the markets are not directly intended for diagnostic purposes and many of them are focused on analysis of hot spots only (Juan *et al*., 2008). In the present study we used a comprehensive mutation assay and analyzed all the four coding exons of *K ras* gene.

MATERIALS AND METHODS

The study was approved by the Ethical Committee of School of Biological Sciences, Lahore and Advanced Board of Studies and Research, University of the Punjab, Lahore, Pakistan.

A 45 year old male was registered in Services Hospital, Lahore, Pakistan with the complaint of moderate abdominal pain and occasional bleeding in the stool. Patient was interviewed and general information about age, gender, nationality, life style, economic condition, dietary habits, family history, smoking habit, presence of any type of addiction, presence of any type of tumor and other health problems were recorded.

Initial computed tomography (CT) scan indicated the presence of a growth in descending colon. Biopsy was performed to address the histological nature of the disease and colonic carcinoma was confirmed. Tumor stage, involvement of lymph node and metastatic stage was investigated. In order to use an anti cancer therapy, it was decided to assess the mutational status of *K ras*. An informed consent was signed by the patient for the participation in the study. A small piece of colorectal tumor tissue, its adjacent normal tissue (12 cm away from the tumor location) were excised during the surgery and immediately snap frozen in liquid nitrogen. The whole blood sample of 5 ml was also taken.

Extraction of genomic DNA

Extraction of genomic DNA from both tumor and normal fresh frozen colonic tissue samples was done using Puregene DNA extraction kit (Gentra Systems) by following the protocol given by the manufacturers. Extraction of genomic DNA from blood samples was done by following the protocol of Helms (1990).

Denaturing gradient gel electrophoresis (DGGE)

For comprehensive mutation assay (which covers all intron-exon boundaries), *K ras* was analyzed for mutations on Denaturing Gradient Gel Electrophoresis (DGGE). Protocol of Hayes *et al*. (2000) with some modifications was followed. A nested PCR was done by using two sets of primers; external primers and internal primers. A GC clamp (GC rich fragment) was added to the 5'-end of one of the primers in each primer set of internal primers (Hayes *et al*., 2000).

In the first round of PCR amplification, 50ng of genomic DNA was used as a template in 50µl reaction mixture, containing 0.2 units of Taq DNA polymerase (Fermentas Life Sciences), 1.5mM $MgCl_2$, 2.5mm dNTPs, and 20pmole of each

external primer. Initial denaturation was carried out for 5 minutes at 94°C, then 35 cycles, each of denaturation at 94°C for 45 seconds, annealing for 60 seconds at specific annealing temperature of each exon (exon 1, 2 and 4 at 54°C, 3 at 52°C), followed by extension at 72°C for 1.15 minutes. The amplified PCR products (10 µl) were checked on 1.5% agarose gel.

In the second round of amplification, 1µl of respective external amplified PCR product was taken as template in a 50µl reaction mixture, containing 0.2 units of Taq DNA polymerase (Life Sciences, Fermentas), 1.5mM $MgCl_2$, 2.5mm dNTPs, and 20 pmole, of each internal set of primer. Initial denaturation was carried out for 5 minutes at 94°C, then 35 cycles each of denaturation at 94°C for 45 seconds, annealing for 60 seconds at 55°C, followed by extension at 72°C for 1.15 minutes. The amplified PCR products (10 µl) were checked on 1.5% agarose gel and visualized under UV transilluminator.

The PCR products were electrophoresed in 9% polyacrylamide gel (acrylamide:bis; 37.5:1) with 20%-60% urea/formamide on DCode Mutation Detection System by Bio-Rad, (model 475). Gel was run parallel to the direction of electrophoresis at 120 V and 59ºC for overnight, stained with ethidium bromide and visualized using UV transilluminator. The stained gels were carefully analyzed and the sample showing any type of mobility shift was further proceeded for sequencing.

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

RESULTS

No family history of CRC was found in the studied subject. The histological evaluation showed a moderately differentiated adenocarcinoma of mucinous type in T3 N0 M0 stage, indicating the invasion of tumor to the muscularis propria of colon (a deeper, thick layer of muscle that contracts to force the contents of the intestine along) along with its metastatic involvement in four or more regional lymph nodes. All the coding regions of gene were amplified and after purification, when all amplified exons were subjected to DGGE analysis, mobility shift was observed in exon 1 only.

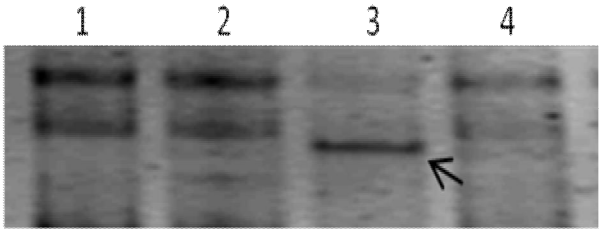

Fig. 1. PCR-DGGE profile on 12 % (Acrylamide: bisacrylamide) for *K ras* exon 1. Lane (1) Healthy control; (2) Patient's blood sample (3) Patient's tumor sample; (mobility shift is visible and denoted with arrow) (4) Patient's normal tissue sample.

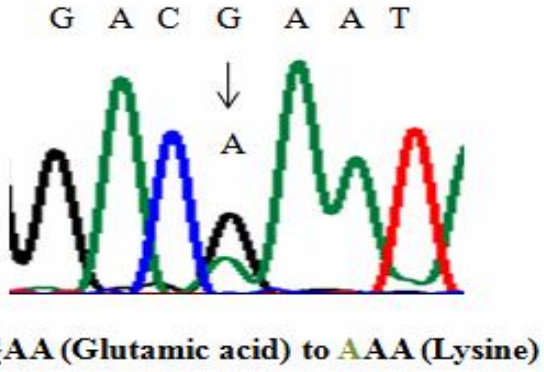

Fig. 2. Sequence analysis of *K ras* exon 1of patient studied, showing G to A transition at the first base of codon 31 resulting in a heterozygous mutation (GAA (glutamic acid) to AAA (lysine).

Sequence analysis revealed the presence of a heterozygous mutation (GAA (glutamic acid) to AAA (lysine) at codon 31. This specific sequence variant was present in a small subclone of tumor cells and only detectable in fresh tumor tissue sample and was not found in normal tissue or blood of the respective patient. Mutant allele showed slightly lower intensity as compared to the wild type. To the best of our knowledge, there are no previous reports about this specific type of mutation in CRC, hence it is a novel variant. No mutation was

found at codon 12 and 13 and 61 which are usually considered as hot spots for the mutation in *K ras*. Furthermore the mutant sample was also analysed for the presence of any mutation in exons 5, 6, 7, 8 and 9 of *p53,* but no mutation was found.

DISCUSSION

K ras mutation spectrum has been studied in different cancers in western populations, but there is very little data for developing countries. Non-canonical *K ras* mutations have also been reported in many studies and may account for transformative capacity by biochemical and signaling readouts, so the functional consequences of these dysregulation should also be characterized. In the current study, we have found a heterozygous mutation at codon 31 substituting glutamic acid (GAA) to lysine AAA in moderately differentiated mucinous adenocarcinoma of invaded to the muscularis propria of colon. Its functional activity and association to CRC needs be clarified by further studies.

Some of the chemical agents for example, N-nitroso compounds which are produced in the processing of red and processed meat, could induce G to A transitions (Bingham *et al.*, 1996)**.** Two possible mechanisms, that could explain this phenomenon are the formation of guanine-adducts in the DNA and the silencing of the O^6-methylguanine DNA methyl-transferase (DNA repair protein which can remove adducts from the O^6 position of guanine) (Toft and Arends, 1998).

Akagi *et al*. (2007) identified a novel G to T transversion at the third position in codon 19 (TTG) which substituted phenylalanine for leucine with high oncogenic activity and active Ras-GTP levels. Naguib *et al*. (2011) have also reported the strong functional transforming activity of combined mutations in codons 19 and 20. Edkins *et al*. (2006) have identified a missense somatic mutation (Ala146Thr) at codon 146, a highly conserved residue in the guanine nucleotide binding domain. Wang *et al*. (2003) identified a novel mutation in the codon 15 which showed decreased GTPase activity in CRC.

Miyakura *et al*. (2002) have identified CAG (glutamine) to CGG (arginine) substitution at codon 22. Dietary factors may also modify the growth of tumors harboring specific *K ras* mutations. This factor with high consumption of refined grain is a dietary pattern directly associated with increased CRC risk (Slattery *et al*., 2000). It has been studied that diet-related carcinogens, such as heterocyclic amines from heavily cooked meat may induce *K ras* mutations and intake of fruits, vegetables or antioxidants lower the risk of CRC (Palli *et al*., 2000). Besides this, less physical activity (Boyle and Langman, 2000), alcoholism (Brooks and Theruvathu, 2005), air pollution (Goldberg *et al*., 2001) and smoking (Bu-Tian *et al*., 2006) have also been proven to be the possible risk factors for CRC.

As the CRC patients harboring *K ras* mutation do not get benefit from the administration of anti-EGFR agents, therefore, testing *K ras* mutations should be taken into account before selecting any anti cancer therapy for the CRC patients. Routine mutation testing of complete coding region of the gene at the time of initial diagnosis of stage II and III tumors should be considered. There is an urgent need to establish and implement clinical practice guidelines and standardized procedures for *K ras* mutation testing in patients with CRC.

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CONFLICT OF INTEREST

All authors state that there is no conflict of interest.

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