Significance and Incidence of KRAS Mutations in Colorectal Carcinoma and Precancerous Lesions-A Retrospective Study in a Tertiary Care Centre

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Abstract

Introduction: KRAS mutation occurs in colorectal cancers in about 35%-45% cases. Colorectal carcinoma develops from precursor lesions and KRAS mutations occur early in carcinogenesis. KRAS mutation status can help to predict the course and also determine optimal adjuvant therapy.

Aims and Objectives: To detect the presence of KRAS mutations in the deoxyribonucleic acid (DNA) isolated from tissue of colorectal carcinomas and precursor lesions and infer the rate of positivity and negativity of these mutations and correlate them with the type and grade of lesion.

Materials and Methods: This was a retrospective hospital based study done in three years (2009 to 2012) at MGM Hospital, Warangal. Tissue from precancerous lesions and diagnosed cases of colorectal carcinomas was studied for histopathology; and for KRAS mutation by PCR-SSCP.

Observations and Results: On histopathology, there were 35 cases of adenocarcinomas, 21 (60%) being well differentiated, 9 (25.7%) and 3 (8.5%) being moderate and poorly differentiated and 2 cases (5.7%) being mucinous carcinoma. PCR-SSCP was performed on 32 cases. 9 cases (33.3% adenomatous polyps and 38% adenocarcinomas) were positive. The KRAS mutation positivity was 50% for poorly differentiated colorectal adenocarcinoma and was 33% each for both well and moderately differentiated adenocarcinoma.

Conclusion: In our study, 38% of colorectal adenocarcinomas and 33.3% of adenomatous adenomas were positive for KRAS mutation. As KRAS has a role in adenoma-carcinoma sequence, and also considering the treatment aspects, we recommend KRAS mutation study for above lesions.

Keywords: Adenomatous polyps, Colorectal adenocarcinomas, KRAS mutation, PCR-SSCP.

Introduction

The KRAS (Kirsten Rat Sarcoma viral oncogene homolog) gene encodes a G-protein involved in the coupling of signal transduction from surface receptors.(1) It is part of the epidermal growth factor receptor (EGFR) signaling cascade, and can undergo activating mutations in approximately 35%-45% of the colorectal carcinoma (CRC) cases. (2) This has been well-established in numerous randomized controlled trials and single-arm trials.(3,4) Various authors have reported that KRAS mutations occur as an early event in the development and progression of colorectal cancers and such tumors have more advanced stage, increased metastatic potential and poor prognosis.(5-7)

KRAS mutation status may play an important role in therapeutic decisions for colorectal cancer patients. Several studies have reported that KRAS mutations confer resistance to anti EGFR monoclonal antibodies.(8-10) On the basis of these data, the European Medicines Agency (EMEA) has approved the use of cetuximab and panitumumab for the treatment of metastatic colorectal cancer having wild type KRAS.(11)

Aims and Objectives

1. To detect the presence of KRAS mutations in the DNA obtained from colorectal carcinomas and its precursor lesions.
2. Infer the rate of positivity and negativity of KRAS mutations and to correlate them with the type of lesion and grade of tumor.
3. To indicate the prognostic and predictive value of KRAS mutations in colorectal carcinomas.

Materials and Methods

This was a retrospective hospital based study carried out over a period of three years (2009 to 2012) in the department of Pathology, MGM Hospital, Warangal in collaboration with Biogene Quest, a research laboratory in Hyderabad.

Inclusion criteria

1. Samples with definite histopathological diagnosis on endoscopic biopsies and resection specimens of colorectal adenocarcinoma were considered.
2. Premalignant lesions included all polyps and non-neoplastic lesions with concomitant mild dysplasia and above.

Exclusion criteria

1. Inadequate samples were excluded.
2. Non-neoplastic lesions without dysplasia.

Specimen Handling: Colorectal biopsies and the resection specimens were fixed in 10% formalin and

were submitted for routine histopathological processing. Sections were cut at five microns for routine hematoxylin and eosin staining (Hand E). After histopathological diagnosis of the lesion was made, the paraffin blocks of the samples were collected.

1. Two sections of 20 microns were taken for DNA isolation for detection of KRAS mutation in the sample.
2. The results were recorded for individual cases.

Fig. 1: Gross specimen of resected large intestine with exophytic growth

KRAS mutation detection by PCR-SSCP: For each case, the H and E slide was examined and representative area was circled. The corresponding area on the 20-micron section slide was then scraped into a labelled 1.5 ml sterile micro tube using a sterile scalpel blade.

DNA isolation was done by Standard non-heating enzymatic digestion method DNA Extraction Protocol and the quality of the DNA extracted was checked by 2% agarose gel electrophoresis and viewed under ultraviolet illuminator after diethylamine staining of the gel.

Amplification of the DNA by PCR: PCR reaction was done for 21 cycles.

Each cycle had these conditions: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s.

PCR-SSCP technique (single strand conformation polymorphism): Amplification of specific DNA sequences by the PCR technique using primers of KRAS exon 1,162 bp was done. PCR reaction was done for 21 cycles. Each cycle had these conditions: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s.

Single strand conformation polymorphism (SSCP) and silver staining: A 10 µl of PCR product was mixed with 20 µl of loading solution containing 18 micro litre of stock form amide (stock form amide 5.9 ml ultrapure form amide and 0.4 ml of 0.5 M EDTA, stored at 4°C) and 2 micro litre 103 two colour dye (0.42 gm xylene cyanol, 0.167 gm bromophenol blue, 10 ml glycerol and 10 ml ddH20). Samples were denatured at 90–95°C for 5 min in a heat block, rapidly cooled to 4^0 C and 25- µl samples were then loaded on a 12% polyacrylamide gel. A vertical gel was used maintained at constant temperature of 21°C. The gels were electrophoresed for 2.5 hours followed by heat denaturation and polyacrylamide gel electrophoresis (PAGE). The presence of at least one altered base in the amplified fragment may induce structural changes of the single strand DNA obtained by denaturation, detectable by differential mobility into the polyacrylamide gel. If the mutated and wild-type sequences are amplified by PCR; then, distinct patterns of electrophoretic migration of both sequences may be observed on the polyacramide gel. Gels were then fixed in 40% methanol and sample bands were visualized by silver staining with silver nitrate staining solution. Positive and negative controls were submitted for each batch of cases.

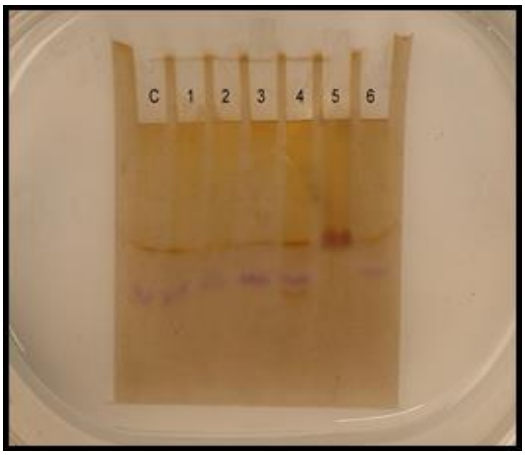

Fig. 2: KRAS by PCR-SSCP, positive in lane 4

Interpretation

The wild type of KRAS amplified fragment gives only one band. The mutated type of KRAS amplified fragment gives polymorphism of band, or more than two bands on the gel, because the mutated DNA sequence has an altered electrophoretic mobility due to conformational change after denaturation.

Observations and Results

In the present study, we evaluated a total of 65 patients for colorectal biopsies and colorectal resections. The patient age ranged from 21 to 73 years. There were 44 males and 21 females and the male to female ratio was 2.09:1.

Table 1: Age distribution of patients

Diagnosis	Age in years						Total
	21-30	31-40	41-50	51-60	61-70	71-80	
Nonsp Colitis	1	6			1		8
SRUS		1	3	1			5
TB	1	1	2				4
Crohns		2					2
UC		2	1				3
Juvenile polyp	1						1
Hyperplastic polyp		3					3
Adenomatous polyp		1	3				4
AdenoCa, well diff			1	4	13	3	21
AdenoCa, Mod diff				6	3		9
AdenoCa, Poorly diff				2	1		3
AdenoCa, Mucinous				1	1		2

SRUS-Solitary rectal ulcer syndrome.
UC-Ulcerative colitis.

Colorectal lesions-microscopic findings: Out of total 65 patients, there were 35 cases (53.8%) of adenocarcinomas. 31 cases of carcinomas (47.69%) were present in the age groups of 51-70 years.

Among the adenocarcinomas, 21 (60 %) were well differentiated adenocarcinomas, 9 (25.7 %) were moderately differentiated, 3 (8.5 %) were poorly differentiated and 2 (5.7 %) were mucinous carcinomas.

13 cases out of 21 (62%) well differentiated adenocarcinomas were in 61-70 age group. 6 cases out of 9 (66.6%) moderately differentiated adenocarcinomas were in 51-60 age group.

KRAS positivity in colorectal lesions: Cases with frank carcinoma and lesions having at least mild dysplasia and above were selected for KRAS mutation study. DNA was successfully isolated from 32 cases and PCR-SSCP was performed on these 32 cases.

Table 2: Cases submitted for KRAS mutation study

Diagnosis	No. of cases	Positive
Hyperplastic polyp	1	-
Adenomatous polyp	3	1
Adeno Ca, well diff	12	4
Adeno Ca, Mod diff	6	2
Adeno Ca, Poorly diff	2	1
Adeno Ca, Mucinous	1	1
Non-sp colitis	2	-
TB	2	-
UC	1	-
SRUS	2	-
Total	**32**	**9**

SRUS- Solitary rectal ulcer syndrome.
UC- Ulcerative colitis.
Total 21(21/35) cases of colorectal adenocarcinomas and three (3/4) adenomatous polyps were submitted for mutation detection.

Results of KRAS mutation detection by PCR-SSCP: Out of 32 cases, 9 cases (1 adenomatous polyp and 8 adenocarcinomas) were positive. None of the non-neoplastic lesions were positive in our study. Total 8 out of 21 cases (38%) of adenocarcinomas were positive for KRAS mutation and 13 cases (62%) were negative. For adenomatous polyps, 1/3 case (33.3%) was positive for KRAS mutation. The KRAS mutation positivity of colorectal adenocarcinomas located in rectum, left colon and right colon was 17% (1/6), 55.5 % (5/9), and 33.3% (2/6) respectively. The KRAS mutation positivity of well differentiated, moderately differentiated and poorly differentiated colorectal adenocarcinomas was 33.3 % (4/12), 33.3% (2/6), and 50% (1/2) respectively. Only one case of mucinous adenocarcinoma was present and it came positive for KRAS mutation.

Discussion

The oncogenesis and development of most colorectal cancers start from normal epithelia and progresses through adenoma and then to adenocarcinoma, and finally to metastasis. This complicated process has been found to involve several oncogene changes in a certain order, that is, from adenomatous polyposis coli (APC) to KRAS to p-53 to deleted in colon cancer (DCC) genes.[12] p-53 and KRAS gene mutations have been observed in both adenomas and carcinomas of large intestine.[13]

Activating mutations in KRAS are a good predictor of CRC as they confer resistance to EGFR targeted monoclonal antibodies. At present it is recommended that all patients with CRC should undergo testing for KRAS status. This will help to categorize patients as only those having wild type KRAS benefit from monoclonal antibody therapies. This will also improve outcomes, and minimize the treatment cost as well as drug toxicity.[14]

Reimers et al.[15] in their study have reviewed the prognostic and predictive biomarkers in colorectal carcinomas such as mutant KRAS, mutant BRAF, and microsatellite instability (MSI) assays and have observed their usefulness in treatment of metastatic colorectal carcinoma, in evaluation of Lynch syndrome and in treatment planning of advanced CRC. Considering the role of KRAS mutation status as an important predictor for treatment response, in present day clinical practice, it is helpful to detect KRAS mutations.[16]

Table 3: KRAS mutation detection rates in colorectal cancers in various studies

Study	No. of cases	KRAS mutation positivity	Method employed
Ahnen et al.[17]	227	90 (40%)	PCR-SSCP, followed by direct sequencing of abnormal SSCP patterns
Tortola et al.[18]	132	54 (41%)	PCR-SSCP, RFLP and sequencing
Zhi-Zhong Pan et al.[19]	97	61 (62.9%)	PCR-SSCP
Morrin et al.[20]	52	19 (36%)	Digoxigenin-labelled cDNA probe by non-radioactive in- situ hybridisation
Akkiprik et al.[21]	43	5 (12%)	PCR-SSCP, followed by direct sequencing of abnormal SSCP patterns
Chiang et al.[22]	57	27 (46%)	PCR-SSCP, RFLP, loss of heterozygosity analysis
Moroni et al.[23]	31	10 (32%)	Sequencing
Amado et al.[24]	427	183 (43%)	Allele specific PCR
Van Cutsem et al.[25]	540	194 (36%)	Melt –curve analysis
Jervoise et al.[26]	2721	1025 (37.7%)	PCR-SSCP, Allele specific PCR, Sequencing
Present study	21	8 (38%)	PCR-SSCP

Table 4: Comparison of clinicopathological parameters in KRAS positive colorectal cancers

		Okulczyk et al.[26]		Present study	
Feature		**No. of cases**	**KRAS mutation**	**No. of cases**	**KRAS mutation**
Sex	Male	30	10 (33.3%)	16	6 (37.5%)
	Female	33	13 (39.4%)	5	2 (40%)
Age	<60years	17	6 (35.3%)	9	3 (33.3)
	>60 years	46	17 (36.9%)	12	5 (41.6%)
Location of tumor	Rectum	30	10 (33.3%)	6	1 (16%)
	Left colon	19	9 (47.3%)	9	5 (55.5%)
	Right colon	14	4 (28.6%)	6	2 (33.3%)
Histologic grade	G1	5	2 (40.0%)	12	4 (33.3%)
	G2	42	15 (35.7%)	6	2 (33.3%)
	G3	14	5 (35.7%)	2	1 (50%)
	Adenocarcinoma	56	19 (33.9%)	20	7 (35%)
	Mucin secreting	7	4 (65.3%)	1	1 (100%)

Okulczyk et al.[27] studied the incidence of KRAS gene mutations in tumour and surgical margins in 63 patients with adenocarcinomas of varied clinical stage and histological grade and detected point mutations in codon 12 KRAS gene using the PCR-RFPL technique in cancer tissue in 23 patients (36.5%). No correlation was observed in their study with either Dukes or TNM clinical advancement.

In the present study, we studied the incidence of KRAS gene mutations in tumor tissue of 21 patients with colorectal adenocarcinomas of varied histological grade. We detected KRAS gene mutations on exon 1 using PCR-SSCP in 8 cases (38%).

Tumours located in the left colon, and mucinous neoplasms displayed a higher incidence of mutations in the present study as compared to the study by Okulczyk et al.[27] In our study, equal incidence was seen with the grade of the tumor. Thus our results are comparable with this study which was done in Poland. The incidence rates of KRAS mutations are also the same in our study population.

Incidence of KRAS mutations in the polyps of colon and rectum in various studies: Chan et al.[28] studied sixty nine serrated polyps, out of which 15 cases (21.7%) were positive for KRAS mutations. Out of these 15 cases, 9 were hyperplastic polyps and 6 were adenomas.

In our study, out of four cases (one hyperplastic and three adenomatous polyps) submitted for KRAS mutation, one adenoma (25%) was positive for KRAS gene mutation. The positive case of adenomatous polyp showed high grade dysplasia on histopathology.

Jass et al.[29] detected KRAS mutations in 4% (2/49) hyperplasic polyps, in 27% (4/15) serrated adenomas, 35% (11/61) of tubular adenomas, and 50% (11/22) of villous adenomas. In our study, 25% (1/4) adenomas were positive for KRAS mutation.

Conclusion

In our study, we noted KRAS positivity in 38% of total colorectal adenocarcinomas and in 33.3% of adenomatous adenomas. As KRAS is known to have a role in adenoma-carcinoma sequence, and also considering the treatment aspects, we recommend testing of colorectal adenocarcinomas and adenomatous adenomas for KRAS mutation.

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