Detection of Kras Gene in Colorectal Cancer Patients through Liquid Biopsy: A Cost-effective Method

Hafiz Syed Muhammad Osama Jafri, Shamim Mushtaq and Saeeda Baig

Department of Biochemistry, Basic Health Sciences, Dr. Ziauddin University, Karachi, Pakistan

ABSTRACT

Objective: To detect the K*ras* gene through liquid biopsy, a less invasive technique in diagnosed colorectal cancer patients.

Study Design: Cross-sectional study.

Place and Duration of Study: Department of Oncology, Dr. Ziauddin Hospital and *Bait-us-Sukoon* Cancer Hospital, Karachi, from 2019 to 2020.

Methodology: Circulating tumor DNA (ctDNA) in colorectal cancer patients was extracted through magnetic bead technique using MagMAX cell free DNA kit (Thermofisher, Uk). The frequency of K*ras* gene was quantified using a real-time polymerase chain reaction (RT-PCR) assay (qPCR). ANOVA and Chi-square tests were utilised for statistical analysis.

Results: Mean threshold cycle (CT) of K*ras* gene showed significantly higher expression 15.6 ± 1.82 (p=0.001) in stage IV CRC cases compared to early stages (19.53 ± 18.223.7 ± 2.9 and 19.8 ± 2.69 of stage 1, 2 and 3, respectively. Similarly, ΔCT mean of *Kras* gene at stage IV showed significantly higher expression of 2.48 ± 1.40 (0.048), compared to 2.39 ± 0.6, 3.12 ± 0.68 and 3.15 ± 0.41 of stage 1, 2 and 3, respectively. Males (n=40, 55%) showed significant association (p=0.001) with CRC compared to females (n=33, 45%). Categorisation of tumor types within different age groups revealed that colon cancer was more frequent (n=11, 15.1%) in the 41-50 age group, while rectal cancer was more frequent (n= 11, 15.1%) in the 41-50 age group, while rectal cancer was more in the 51-60 age group (n=11, 15.1%).

Conclusion: K*ras* gene was detected with significantly increased levels in plasma of CRC patients at advanced stages. This confirms that liquid biopsy can be used to detect K*ras* gene in ctDNA of CRC patients through a magnetic bead based technique.

Key Words: *Liquid biopsy, Circulating tumor DNA, KRAS, Colorectal cancer, Real-time polymerase chain reaction.*

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INTRODUCTION

Colorectal cancer (CRC) globally is one of the main stream cancer causing mortalities in both genders being the 7th most common cancer in women, while 4th in men according to Globacan statistics with low prevalence in Asia and Africa.[1] Colorectal cancer is a highly diversified, heterogeneous and complex disease containing cells of the same histological type with different genotypes or cell subtypes.[2] Effective monitoring and management are, therefore, extremely essential for the regular evaluation of the disease.

K*ras*, a proto-oncogene, having dual function, plays a major role both in inhibition of cancer as well as in carcinogenesis.

Correspondence to: Prof. Dr. Saeeda Baig, Department of Biochemistry, Basic Health Sciences, Dr. Ziauddin University, Clifton, Karachi, Pakistan
E-mail: saeeda.baig@zu.edu.pk

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Mutation of K*ras* on codons 12, 13 transforms it into an oncogene, identified in the colorectal cancer (CRC; 25-45%), lung cancer (35%), and breast cancer (5-10%).[3] K*ras*, a prominent member of the Ras family, plays a major role in healthy tissue signalling through cycling of GTPase between active GTP, K*ras* GTP and K*ras* GDP, which is inactive formations. Mutations in K*ras* can impair GTPase's function, leading to permanent activation of AKT / mTOR / PI3K and MEK / ERK / RAF, the downstream signalling pathways. Thus, the ongoing activation of K*ras* ultimately develops into malignancies.[4]

Liquid biopsy, an outstanding molecular diagnostic method, which can detect tumor DNA fragments, known as circulating tumor DNA (ctDNA), floating in the blood (plasma) with minimal invasiveness and is highly responsive tool for CRC patients. ctDNA is found in a number of body fluids, including blood plasma, synovial fluid, and cerebrospinal fluid, originating from cancer cells that have undergone cell death; it is composed of single or double stranded DNA.[5] The ctDNA has increasingly progressed from research to clinical use as the first option for liquid biopsy, because its testing is less invasive and replicable/reproducible,[6] detecting gene mutations in a convenient manner. In addition, another theoretical benefit of liquid biopsy

is in case of recurrence or relapsed of disease; a thorough overview of the genetic map of the disease can be obtained.[7] The ctDNA is widely used prior to clinical and radiological confirmation to recognise measurable genomic changes, assess treatment responses, unravel therapeutic resistance, and potentially detect disease progression through tumor monitoring and oncotherapy.[8] Liquid biopsy corresponds to a possible option for K*ras* testing, because tissue samples may not always be available.

The purpose of this study was to detect K*ras* gene in colorectal cancer patients through liquid biopsy.

METHODOLOGY

A total of 73 diagnosed patients of primary colorectal carcinoma (Stage I, II, III, IV) were enrolled from the Department of Oncology, Dr. Ziauddin Hospital Karachi, Pakistan, from 2019 to 2020. The study was approved by the Ethical Review Committee (ERC) of Dr. Ziauddin University. It was a cross-sectional study. Non-probability consecutive sampling technique was used. All diagnosed CRC patients were enrolled in the study. Multiple primary cancers and HNPCC/Lynch syndrome patients were excluded from the study. After written informed consent, patients were interviewed and complete demographic data were recorded as well as lifestyle, dietary habits, past medical history, family history, bowel habits, smoking history, presence of any other type of tumor, were also recorded. Blood samples (3-5 ml) were drawn from the patients. After centrifugation, plasma was separated and ctDNA was extracted out from plasma.

Circulating tumor DNA was extracted from 200 µL plasma samples through magnetic bead-based technique using MagMAX cell-free DNA kit, catalog No. A29319 (Thermo Fisher, UK), according to the manufacturer's instructions. The extracted ctDNA was stored at -20C.

ctDNA concentrations in all plasma samples were measured using the RT-PCR assay SLAN -48P RT-PCR System (Shanghai, China) and qPCR master mix (BlasTaq™ 2X qPCR Master Mix Cat. No. G891). The K*ras* primers were: Forward Primer: 5-CGATACACGTCTGCAGTCAAC-3, Reverse Primer: 5-ACCCTGACATACTCCCAAGGA-3. The housekeeping gene GAPDH Forward Primer: 5-ACCCACTCCTCCACCTTTGAC-3, and Reverse Primer 3-CTGTTGCTGTAGCCAAATTCG-5 were used as internal control. Amplifications were performed for 42 cycles (95 °C for 5 minutes, 1 cycle; 95 °C for 15 s, 60 °C for 1 minute, and 25 °C for 10 seconds). CT values were analysed and interpreted accordingly. SPSS version 20 was used for data analysis. ANOVA and Chi-square tests were utilised. At 95% confidence level, p value of less than 0.05 was considered as statistically significant.

RESULTS

In this study, out of 73 colorectal cancer patients, (n=40, 55%) were males and (n=33, 45%) were females (p=0.001) showing a significant association of CRC with males, with mean age of 46.25 ± 11.32 years. Among the ethnicities, Urdu speaking and Punjabi showed significant association with colorectal cancer (p=0.001) in this study population. Other demographical details are given in Table I.

Table I: Demographical data.

	Variables	Value n (%)
Age (year)	Mean ± SD	46.25 ± 11.32
Gender	Male	40 (55)
	Female	33 (45)
Ethnicity	Sindhi	15 (20)
	Punjabi	17 (23)
	Pathan	12 (16)
	Balochi	12 (16)
	Urdu speaking	17 (23)
Marital status	Unmarried	10 (13.5)
	Married	63 (85)
BMI status	Normal	21 (28)
	Overweight	15 (20)
	Underweight	37 (50)

Categorisation of CRC types within different age groups revealed that colon cancer was more frequent (n=11, 15.1%) in 41- 50 year age group, and rectal cancer was more frequent in the 51-60 year age group (n=11, 15.1%). Colorectal cancer was not detected in the age groups of 20 -30 or 61-70 year (Figure 1).

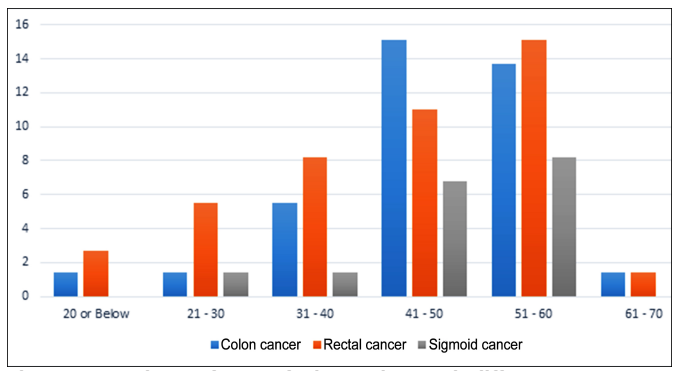

Figure 1: Prevalence of types of colorectal cancer in different age groups.

RT-PCR was performed to detect K*ras* gene in colorectal cancer patients. Relative quantification of target K*ras* gene shows higher (upregulated) values in late stages (stage III and IV) compared to earlier stages (I and II). In stage IV, lowest threshold cycle (CT value) *i.e.* 12.98 were observed in 41.1% CRC patients, demonstrating higher ctDNA load; while in stage I, CT load was found to be 33.40 in 9.6% CRC patients. These findings revealed that the ctDNA concentration was higher in late stages compared to earlier stages. There were significant differences of CT value among the stages of CRC *i.e.* p=0.001 as shown in Table II. However, results also revealed significant difference among stages of CRC in terms of ΔCT (p=0.048), while ΔΔCT and fold change are insignificant among different stages of CRC.

DISCUSSION

The lowest CT value of 12.98, in the current study, was observed in 41.1% of stage IV CRC patients, demonstrating higher ctDNA load compared to early stages of CRC. These findings disclose that the ctDNA concentration is higher in later stages compared to early stages.

Table II: Minimum and maximum CT values for cancer stages, CT and ΔCT Mean of Kras gene, and *p*-values for colorectal cancer patients. *Significance level (p-value <0.05), the mean CT and ΔCT difference of K*ras* gene are significant at the 0.05 level. One Way ANOVA is applied.

Stages	Stage I	Stage II	Stage III	Stage IV	p-value
Minimum CT value	.00	17.36	13.87	12.98	
Maximum CT value	35.32	30.1	23.57	19.34	
Mean CT ± Std.	19.53±18.2	23.7±2.9	19.8±2.69	15.6±1.82	0.001*
ΔCT Mean ± Std.	2.39±0.6	3.12±0.68	3.15±0.41	2.48±1.40	0.048*

However, mean ΔCT was significantly lower in the earlier stages to advanced stages of colorectal cancer. The possible explanation for upregulation of K*ras* gene in ctDNA is due to the fact that in cancer patients ctDNA levels are stable and differ greatly, from 0.01% to more than 90%, according to cancer stage. However, cell-free DNA is present in healthy individuals as well; but is quickly phagocytised. However, ctDNA variability correlates with tumor burden, vascularity, cellular turnover, and response to therapy in cancer patients.[9] A previous study displayed a substantial spike in ctDNA concentrations in early stages of the disease (TNM 0-II) compared to healthy individuals. These researchers also observed significantly higher ctDNA in primary CRC than in intestinal polyps and healthy controls in correlation with age, histologic differentiation and the tumor stage.[10] In this study, patients with stage IV cancer had higher expression (lower CT value) compared to the initial stages of cancer.

Various techniques are now available for testing known mutations in ctDNA in CRC. The most popular are digital PCR or quantitative PCR.[11] For the detection of mutation of genes through liquid biopsy (ctDNA), researchers have reported the use of Droplet Digital PCR (ddPCR).[12] However, very few studies are available regarding the use of qPCR for the detection of mutated gene expression from extracted ctDNA. In this study, it was demonstrated for the first time that qPCR is able to detect K*ras* gene in extracted ctDNA from colorectal cancer patients. A study conducted in China reported the possible utility of serum-free circulating tumor DNA as a diagnostic marker to classify patients with CRC and colon polyps.[13] The prognosis of CRC patients is largely based on the stage of the disease at the time of diagnosis.

Liquid biopsy opened a new era in the field of cancer research by detecting ctDNA in the plasma of cancer patients.[14] It is found that necrotic or apoptotic cancer cells are releasing DNA fragments, *i.e.* circulating tumor DNA (ctDNA) into the blood stream.[15] Apoptotic and necrotic cells are cleared by infiltrating phagocytes under normal physiologic conditions and the levels of cell-free ctDNA are relatively low.[9] A study reported that the mean ctDNA index tends to be around 50 times higher for CRC patients than for healthy individuals.[16] While the clinical significance of circulating tumor DNA has yet to be determined thoroughly, several studies have shown that measuring ctDNA in cancer patients' plasma may be a promising method for cancer screening, diagnosis, and therapy monitoring.[17]

In this study, among ethnicities, ctDNA concentration were found to be significantly higher (p=0.001) in Punjabi and Urdu speaking populations. Currently, no data is available on the frequency of colorectal cancer (CRC) within different ethnicities of Pakistan. Disparities in genetics channel to inter-ethnic differences, that can lead to susceptibility to some cancers or diseases.[18] A study reported that rectal cancer incidence has generally been declining in age groups older than 55 years since 1974, in contrast to colon cancer.[19] On the contrary, categorisation of CRC forms within various age groups showed that rectal cancer as well as colon cancer is more prevalent (n=11, 15.1%) in the 51-60 and 41-50 years age groups, respectively. These might be due to the fact that males, especially at later age, have greater tendency to deposit visceral fat, which in turn is associated with increased risk of CRC. Other risk factors for males may include unhealthy eating habits such as diet high in red or processed meat or heavy smoking.[20] There are also different inherent variations and unknown risk factors within the colorectum along with environmental factors, causing initiation and/or promotion of carcinogenesis.

The results were more pronounced in males (n=40, 55%) than in females (n=33, 45%) with ratio of 1.2:1, which is in contrast to a study reported earlier from Pakistan depicting male-to-female ratio as 1.9:1.[21] Another study showed male gender having a global trend of higher incidence (746,298 *vs.* 614,304 [20.6 *vs*. 14.3 ASR]) as well as increased mortality (373,639 *vs.* 320,294 [10 *vs.* 6.9 ASR]).[22] Bowel Cancer Incidence Statistics, CRUK. 2017, also reported that males have more potential to develop CRC with age-standardised rates of 86.1/100,000 compared to 56.9/100,000 female in UK in 2014 (male to female ratio 1.7:1). Likewise mortality rates are also higher in males compared to females aged above 45 years.[23]

Overall, it is demonstrated that ctDNA can be detected through liquid biopsy, as it is useful technique to find K*ras* gene using qPCR assay in CRC patients. It provides a minimally invasive and highly responsive tool for CRC patients to test the K*ras* gene in plasma that can be easily conducted as baseline diagnosis to select patients for personalised therapy.

CONCLUSION

Liquid biopsy is convenient and clinically relevant for doing less invasive biopsies in CRC patients. The magnetic bead--based technique for extraction of ctDNA from plasma samples is accurate and reproducible. RT-PCR is feasible for detection of K*ras* gene in plasma which was significantly higher (upregulated) in primary CRC patients. To confirm the

clinical utility of plasma ctDNA in the diagnosis and prognostic prediction of CRC, further large-scale and longer prospective studies are required.

DISCLAIMER:
The above research paper is a part of M. Phil thesis project titled "Liquid biopsy: A potential tool for detection of Kras gene expression in colorectal cancer patients". It was supervised by Prof. Dr. Saeeda Baig, Co-supervised by Prof. Dr. Shamim Mushtaq and the Clinical Co-supervisor was Dr. Jawed Malik.

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ETHICAL APPROVAL:
The study was conducted after approval from the Ethical Review Committee (ERC) of Ziauddin University, Karachi, Pakistan, dated 28th June 2019, Reference code: 1160519O-JBIO.

PATIENTS' CONSENT:
All patients were informed about the study and a written informed consent was obtained from each patient.

CONFLICT OF INTEREST:
The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
HSMOJ, SB: Designed the project.
HSMOJ: Performed all the experiments.
SM, SB: Participated in its design and coordination and supervision of the project.
SB: Helped in writing and editing the manuscript.
All the authors were involved in drafting the manuscripts and its critical revision; and read and approved the final manuscript for publication.

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