ORIGINAL ARTICLE

ANALYSIS OF COMMON SOMATIC MUTATIONS IN COLORECTAL CARCINOMA AND ASSOCIATED DYSREGULATED PATHWAYS

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Background: Identification of gene targets and biological pathways involved in colorectal carcinoma (CRC) is essential for better management of patients. Our study aims to highlight common somatic mutations in colorectal carcinoma and to identify dysregulated pathways and gene enrichment based on *KRAS* and *BRAF* interaction network analysis. **Methods:** By using cancer browser tool in COSMIC database, mutation frequencies of the top 20 mutated genes listed for colorectal adenocarcinoma were identified. The most frequent variants of selected genes were explored with ClinVar database which led to identification of protein change along with its cytogenic location, variant type, variant length and the associated single nucleotide polymorphism (SNP). These identified SNPs were searched in Pakistani database using 1000genome in an attempt to identify common polymorphisms. Using the database ClinicalTrial.gov the number of clinical trials based upon these selected mutations was explored. Enrichment and protein interaction (PI) analysis of *KRAS* and *BRAF* was carried out to reveal significant biological pathways associated with these genes. **Results:** In cumulative data, among all variants about 57% of substitution mutations are observed to be G>A including mutations in *KRAS, Tp53, SMAD4, PI3K* and *NRAS.* The mutations of *KRAS* (c.35G>A), *TP53* (c.524G>A) and *APC* (c.4348C>T) were found to be pathogenic with single nucleotide variation and variant length of 1bp. Searching 1000genome database revealed that 100 % of alleles found in East Asian population studied are 'C'(frequency=1). Significant biological pathways (<0.05) identified by our search include Trk receptor signalling mediated by the MAPK pathway, signalling to p38 via RIT and RIN, signalling to ERKs, Frs2-mediated activation, ARMS-mediated activation and prolonged ERK activation events. **Conclusion:** Our study highlights the role of genetic profiling in CRC, with emphasis on mutations which may define treatment outcome. Targeting several collateral pathways simultaneously may be further explored to improve colorectal cancer therapeutics.

Keywords: Genetic mutations; Biological Pathways; in-silico; *KRAS*; *BRAF*

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INTRODUCTION

Globally, colorectal cancer (CRC) forms a primary cause of cancer associated morbidity and mortality.[1] Asian CRC has been reported to have the highest proportions of both incident cases as well as mortality cases among all ages and genders.[2] In Pakistan, the prevalence of colorectal cancer has been reported to be about five percent.[3] Unfortunately, about 20% of newly diagnosed CRC patients have metastasis at presentation resulting in increased mortality.[4,5] Although treatment modalities have shown improvements, the five year survival rate in metastatic CRC is only 12–14%.[4] For mCRC surgery, chemotherapy, and irradiation form the main stream therapeutic approaches.[6,7] In advanced cancers, where surgery does not offer cure, clinical approach is mainly focused upon mutation-based targeted therapy. The angiogenic inhibitors ,which inhibit new blood vessel growth, and anti-EGFR monoclonal antibodies, which mainly target the mitogen activated protein (MAP) kinase pathway, have immensely improved the clinical outcomes in metastatic CRC.[8,9] However, anti-EGFR treatment shows poor response in presence of downstream mutations of MAP kinase (MAPK) pathway.[10]

The pathogenesis of CRC is based on diverse molecular events. The main molecular pathways implicated in CRC include the Chromosome Instability (CIN) Pathway signifying sporadic colon cancer, and the Microsatellite Instability (MSI) Pathway, involving mutations in mismatch repair (MMR) genes.MSI pathway represents hereditary non-polyposis colon cancer as well as some sporadic cases.[11] About 80% of CRCs are found to have mutations in CIN pathway which defines adenoma carcinoma sequence emerging through mutations of several genes including KRAS, APC, and TP53 genes.[12] Studies have shown that KRAS mutation is present in about 40% of sporadic CRCs.[13] However, the KRAS mutation alone cannot to lead to malignant transformation. Additional driver mutations, like APC mutation, play a pivotal role in triggering neoplastic changes. Mutated KRAS sends a downstream

signal to B-type RAF proto-oncogene (BRAF) kinase which results in triggering the mitogen-activated protein kinase (MAPK) signaling cascade.[14] Furthermore, KRAS mutations in colorectal carcinoma have been reported to be associated with mutations in genes encoding catalytic subunits of PI3K. The PI3K mutations are arise late in adenoma carcinoma sequence and result in proliferation of colorectal cancer cells by evasion of apoptosis.[15] Since a plethora of genes play a role in causation of CRC a deep insight of molecular aberrations is required to improve patient management. While the world has stepped towards precision medicine, a basic genetic profile is nonexistent in Pakistan. Since our population specific data is lacking, bioinformatics analysis may enable us to discover therapeutically significant mutations and biological pathways. Deeper understanding of genetic alterations in colorectal carcinoma and the functional consequences of these mutations can lead to improved therapeutic approach and better patient management.

MATERIAL AND METHODS

The study has been carried out after approval from Ethics Review Committee of Ziauddin University (2861120SHPAT). The selection of genes studied in our research was based upon 'The Catalogue of Somatic Mutations in Cancer' (COSMIC) database, which is a standardized repository containing somatic mutation data from diverse sources. By using cancer browser tool in COSMIC database, we selected large intestinal tumours and further chose tissue histology as adenocarcinoma. The list of genes presented upon our search showed how many tumors had been examined in each gene and the mutation frequency of the top 20 mutated genes for large intestinal adenocarcinoma. On applying additional filters of 'pathogenic' mutations in 'tumor samples' we identified the variants of the top 20 mutated genes. For each gene, using the total samples with mutations, we explored the variants of each gene. The most frequent substitution mutations were identified. Moreover, the most frequent variants of the top four genes among the list of 20 mutated genes were selected to explore ClinVar database which led to identification of protein change along with its cytogenic location, variant type, variant length and the associated single nucleotide polymorphism (SNP). These identified SNPs were searched in Pakistani database using 1000genome in an attempt to identify common polymorphisms . This was followed by exploring the number of clinical trials based upon these selected mutations using the database ClinicalTrial.gov. We applied filter to include clinical trials which are recruiting, active but not recruiting or completed.

Based on the significant therapeutic implications, *BRAF* and *KRAS* were selected for Enrichment and protein–protein interaction (PPI) network analysis by using Functional enrichment Analysis tool (FunRich) version 3.1.3 March 2017.[16] Hypergeometric test, BH and Bonferroni test were applied in FunRich software. By using the Hypergeometric test and p-value correction with the BH method and Bonferroni tests, significant interactions and pathways associated with datasets were identified. After Bonferri correction, the statistical cut-off of enrichment analyses was kept as p=<0.05. The biological pathways showing significant association with these *KRAS* and *BRAF* were identified.

RESULTS

COSMIC database revealed that out of 54229 large intestinal tumors 47443 were carcinomas. Out of these large intestinal carcinomas, 46924 were adenocarcinoma. The Cancer browser page of COSMIC database presented a list of top 20 mutated genes in which the tumour was examined. The most frequent substitution mutations in each gene with their corresponding position and amino acid mutations was observed (Table-1). It was found that the most common type of mutation is missense substitution which constitutes about 86% of all mutations. In cumulative data, among all variants about 57% of substitution mutations are observed to be G>A including mutations in *KRAS, Tp53, SMAD4, PI3K* and *NRAS.* We narrowed down our search to mutations of the top 4 genes (Table-1) and researched ClinVar database. The mutations of *KRAS* (c.35G>A), *TP53* (c.524G>A) and *APC* (c.4348C>T) were found to be pathogenic with Single nucleotide variation and variant length of 1bp. *BRAF* (c.1799T>A) was found to be interpreted as 'likely pathogenic' with variant type Indel and 2bp variant length. The *KRAS* c.35G>A on searching ClinVar database revealed G12D protein change at cytogenic location of 12p12.1. The associated dbSNP reports rs121913529 at position chr12:25245350 GRCh38.p12. On exploring *Tp53* c.524G>A protein change of R175H R136H, R43H and R16H at cytogenic location of 17p13.1 was revealed. The associated dbSNP reports rs28934578 at position chr17:7675088 GRCh38.p12.For *APC*, the protein change based on nonsense mutations was found to include R1432*, R1450*, R1167*, R1290*, R1422*, R1425*, R1349*, R1359*, R1409*, R1460*, R1324*, R1391*and R1468*. The dbSNP revealed rs121913332 at position chr5:112839942(GRCh38.p12). Upon researching *BRAF* c.1799T>A on ClinVar database, the protein change of V600E is found at cytogenic location of 7q34. The dbSNP revealed rs121913377 is revealed to be at position chr7:140753335-140753336(GRCh38.p12).On searching 1000genome database, it was observed that 100 % of alleles found in East Asian population studied (EAS) are 'C'(frequency =1). The variant rs121913529 (KRAS) has alleles C/A/G/T with ancestral C and

highest population MAF of <0.01. This variant overlaps 4 transcripts and is associated with 34 phenotypes. For variant rs28934578 (TP53) has C/A/T alleles with ancestral C and highest population MAF of <0.01. This variant overlaps 13 transcripts and is associated with 11 phenotypes. The variant rs121913332 (APC) has alleles C/G/T with ancestral C and highest population MAF of <0.01. This variant overlaps 5 transcripts and is associated with 5 phenotypes. The variant rs121913377 (BRAF) has alleles CA/AT/TT with ancestral CA. This variant overlaps 4 transcripts and is associated with3 phenotypes. Upon searching for the identification of these SNPs in our local population it was further highlighted that the patient data is lacking in 1000 genome. To identify clinical trials based on common mutations of CRC, we searched ClinicalTrials.gov database which revealed a total of 5689 clinical trials for CRC. To further narrow our search we applied filter to include clinical trials which are either recruiting, active but not recruiting or completed. The *KRAS* based clinical trials are found to be mostly carried out in European region with 91 out of the 192 registered trials (Figure-1a). Out of the total of 107 *BRAF* clinical trials, 55 are identified to be in Europe while 52 in South America(Figure-1b). Interventional studies based on *APC* and *Tp53* have also been reported though they do not appear to have significant therapeutic implications (Figure-1c & d).

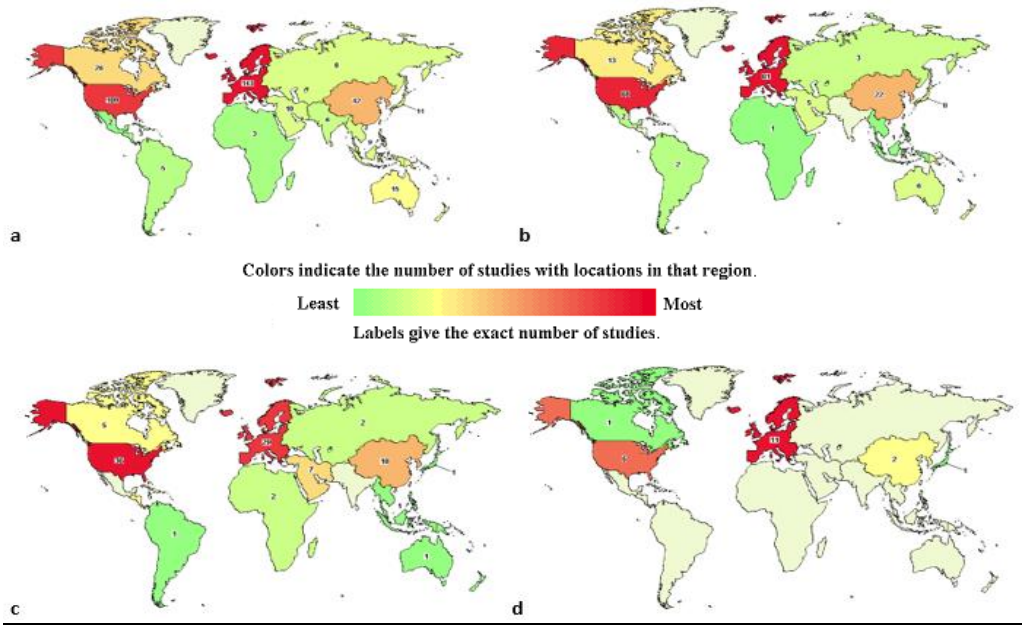

Figure-1: Geographical distribution of number of clinical trials conducted around the world based upon mutation of a=KRAS, b=BRAF, c=APC, d=TP53. The number of trials shown in figure include the clinical trials which are recruiting, are active but not recruiting and the trials which are complete.

Table-1: Genetic variants associated with colorectal carcinoma as reported by COSMIC database

Gene	Variants (n)	Legacy	Position	CDS mutation	AA Mutation	%age	Substitution
KRAS	99	COSM521	12	c.35G>A	p.G12D	35.88	Missense
BRAF	142	COSM476	600	c.1799T>A	p.V600E	57.65	Missense
TP53	525	COSM10648	175	c.524G>A	p.R175H	6.75	Missense
APC	1026	COSM13127	1450	c.4348C>T	p.R1450*	5.91	Nonsense
PI3K	276	COSM763	545	c.1633G>A	p.E545K	20.96	Missense
FBX W7	201	COSM22965	465	c.1394G>A	p.R465H	5.84	Missense
SMAD4	342	COSM14122	361	c.1082G>A	p.R361H	8.95	Missense
LRPB1	326	COSM1236069	3837	c.11511G>A	p.M3837I	0.78	Missense
TCF7L2/tcf4	118	COSM32406	471	c.1411C>T	p.R471C	3.19	Missense
FAT4	234	COSM1050990	3735	c.11203C>T	p.R3735C	0.34	Missense
		COSM9176993	3858	c.11574G>A	p.W3858*	0.34	Nonsense
NRAS	34	COSM564	12	c.35G>A	p.G12D	16.75	Missense
KMT2C	387	COSM1179670	309	c.925C>T	p.P309S	2.71	Missense
CTNNB1	143	COSM5667	45	c.134C>T	p.S45F	16.0	Missense
ATM	259	COSM21323	337	c.1009C>T	p.R337C	3.03	Missense
RNF	67	COSM981870	132	c.394C>T	p.R132*	1.37	Nonsense
		COSM248786	145	c.433C>T	p.R145*	1.37	Nonsense
KMT2D	179	COSM6284332	1194	c.3581C>A	p.P1194H	4.82	Missense
PTEN	135	COSM5154	233	c.697C>T	p.R233*	4.34	Nonsense
ARID1A	134	COSM51425	1989	c.5965C>T	p.R1989*	1.35	Nonsense
POLE	148	COSM937332	286	c.857C>G	p.P286R	4.97	Missense
AMER1	129	COSM28714	353	c.1057C>T	p.R353*	2.73	Nonsense
		COSM26840	497	c.1489C>T	p.R497*	2.73	Nonsense

COSM=catalogue of somatic mutation, *= stop codon

Table-2: Biological Pathways associated with *KRAS* and *BRAF* (*p*=<0.05)

Biological Pathway	Fold enrichment	*p*-value (Hypergeometric Test)	Bonferroni Method	BH method	Genes mapped
Signalling to p38 via RIT and RIN	392.880075	6.06707E-06	0.010119873	0.004005783	*KRAS; BRAF;*
Signalling to ERKs	209.5971343	2.19931E-05	0.036684538	0.007336908	*KRAS; BRAF;*
Frs2-mediated activation	330.879011	8.64557E-06	0.014420819	0.004005783	*KRAS; BRAF;*
ARMS-mediated activation	349.2509717	7.73551E-06	0.012902838	0.004005783	*KRAS; BRAF;*
Prolonged ERK activation events	314.3433283	9.60619E-06	0.016023132	0.004005783	*KRAS; BRAF;*
Trk receptor signalling mediated by the MAPK pathway	184.9458983	2.83636E-05	0.047310405	0.007885067	*KRAS; BRAF;*

RIT= Ras-like protein in tissues, RIN = Ras-like protein in neurons , ERK= extracellular-signal-regulated kinase Frs2= Fibroblast Growth Factor Receptor Substrate 2 ARMS =Ankyrin-Rich Membrane Spanning Trk=tyrosine receptor kinase

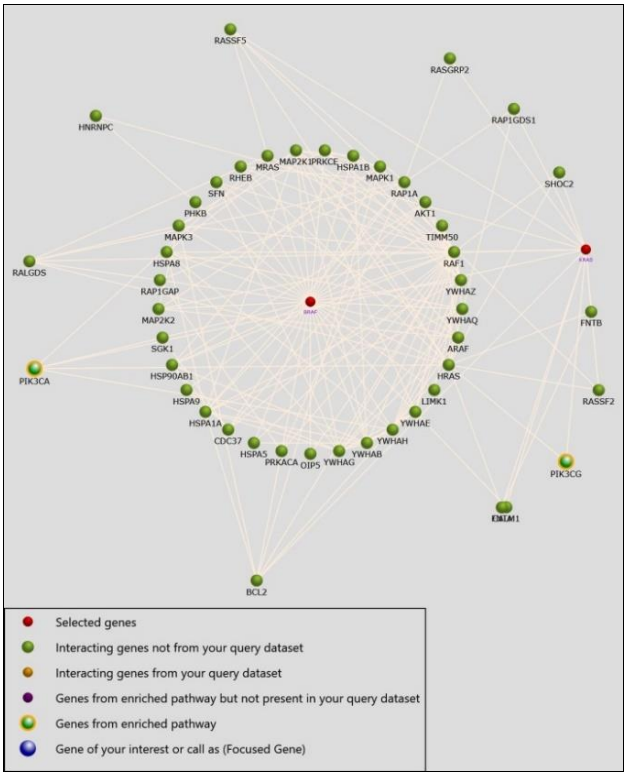

Figure-2: Protein –Protein Interaction (PPI) network of KRAS and BRAF(selected genes)

HNRNPC= Heterogeneous nuclear ribonucleoproteins C, RAP1GDS1=Rap1 GTPase-GDP dissociation stimulator 1, RASSF2= Ras Association Domain Family Member 2, RASSF5= Ras Association Domain Family Member 5, FNTA =farnesyltransferase type-1 subunit alpha, FNTB=farnesyltransferase type-1 subunit beta, PIK3CG= Phosphatidylinositol-4, 5-Bisphosphate 3-Kinase Catalytic Subunit Gamma,RASGRP2=RAS guanyl-releasing protein 2, SHOC2=Leucine Rich Repeat Scaffold Protein, RALGDS=Ral guanine nucleotide dissociation stimulator, RAF1=Rapidly Accelerated Fibrosarcoma/ Serine-Threonine Kinase, BCL2=B-cell lymphoma 2- poptosis regulator, CALM1=Calmodulin 1, PIK3CA=phosphatidylinositol-4, 5-Bisphosphate 3-Kinase catalytic subunit alpha, OIP5=Opa Interacting Protein 5, YWHAG = Tryptophan 5-Monooxygenase Activation Protein Gamma YWHAB= Tryptophan 5-Monooxygenase Activation Protein beta, YWHAH= Tryptophan 5-Monooxygenase Activation Protein Eta, YWHAE= Tryptophan 5-Monooxygenase Activation Protein Epsilon, LIMK1=LIM Domain Kinase 1, HRAS=Harvey rat sarcoma viral oncogene homolog, ARAF=A-Raf protooncogene serine/threonine kinase, YWHAQ= Tryptophan 5-Monooxygenase Activation Protein Theta, WHAZ= Tryptophan 5-Monooxygenase Activation Protein Zeta,TIMM50= translocase of Inner Mitochondrial Membrane 50, AKT1= AKT Serine/Threonine Kinase 1, RAP1A= Ras related protein 1A,MAPK1= Mitogen-Activated Protein Kinase 1, HSPA1B=Heat Shock Protein Family A member 1B, PRKCE=Protein Kinase C Epsilon, MAP2K1 = Mitogen-Activated Protein Kinase Kinase, MRAS= Muscle RAS Oncogene Homolog, RHEB= Ras Homolog, MTORC1 Binding, SFN= Stratifin, PHKB= Phosphorylase Kinase Regulatory Subunit beta, MAPK3 Mitogen-Activated Protein Kinase 3, HSPA8 = Heat Shock Protein Family A member 8, RAP1GAP=RAP1 GTPase Activating Protein, MAP2K2 Mitogen-Activated Protein Kinase Kinase 2, SGK1= Serum Glucocorticoid Regulated Kinase 1, HSP90AB1=Heat Shock Protein 90 Alpha Family Class B member 1, HSPA9 =Heat Shock Protein Family member 9, HSPA1A = Heat Shock Protein Family member 1A, CDC37= Cell Division Cycle 37, HSPA5= Heat Shock Protein Family member 5, PRKACA=Protein Kinase CAMP-Activated Catalytic Subunit Alpha.

FunRich tool was used to perform protein–protein interaction network visualization and analysis of *BRAF* and *KRAS*. The PPI network showed the selected KRAS and BRAF 47 genes all of which showed interaction with eachother (Figure-2). The leading biological pathways (*p*=<0.05) associated with these interacting proteins were found to be Trk receptor signaling mediated by the MAPK pathway, signalling to p38 via Ras-like protein in tissues (RIT) and Ras-like protein in neurons (RIN) GTPases, signalling to ERKs, Frs2-mediated activation, Ankyrin-Rich Membrane Spanning -mediated activation and prolonged ERK activation events (Table-2).

DISCUSSION

Colorectal carcinoma has one of the highest mutational burdens and several somatic mutations have been associated with CRC.[16] Most commonly implicated genes follow CIN pathway and are characterized by chromosome changes that include somatic copy number alterations caused by aneuploidy, deletions, insertions, amplifications, or loss of heterozygosity.[17] The presence of *RAS* mutations confers a worse prognosis in early-stage CRC, with higher chances of relapse and reduced overall survival.[8] *KRAS*-driven cancers are considered to be "undruggable" as they mostly resist therapeutic intervention.[8] Our study reveals c.35G>T substitution resulting in p.G12V amino acid mutation as the most frequent *KRAS* mutation at position 12.The prognostic effect is associated with *KRAS* codon 12 mutations and left-sided MSS (Microsatellite Stable) tumors while in metastatic tumours, survival is reduced in *KRAS*-mutated CRC. More important is their negative predictive value in metastatic CRC, with compelling evidence of primary resistance to antiEGFR mAbs.[18] The codon 12 and 13 *KRAS* mutations were the first to be causally implicated in primary resistance to anti-*EGFR* drugs like cetuximab or panitumumab.[11] Mutation in KRAS genes is an important focus while planning cancer therapy as is associated with resistance to anti-EGFR immunotherapy.[19] While our search identified 5 clinical trials based on *KRAS* mutations in India, there were none revealed in Pakistan. Target therapies which can successfully treat KRAS mutant CRC are unavailable. Even though clinical trials have revealed that inhibitors for the *KRAS* G12C mutation show anti-tumor activity, *KRAS* target therapy is yet to establish. Therefore, understanding biological pathways downstream of *KRAS* and their link to the cancer phenotype needs to be further explored.[20]

KRAS, as a component of epidermal growth factor receptor (EGFR) pathway, leads to constitutive activation of RAF/MEK/ ERK pathway, PI3K signaling via MTOR, and the transcription factor NF-kB.A member of RAF protein family, *BRAF*, may undergo gain of function mutation triggering MAPK pathway.[17] *KRAS* and *BRAF* mutations are considered to be mutually exclusive.[21] Missense mutation at valine 600 residue has been documented constitute 90% of all *BRAF* mutations while they are reported in about 10% of colorectal carcinomas.[7,22] Our search on COSMIC database revealed similar report with about 57 percent mutations of *BRAF* mutations to be missense mutations c.1799T>A at 600 position. *BRAFV600E* mutations seem to play a crucial role in CRC as they have been documented to be associated with poor treatment response and unique metastatic spread.[23] Literature shows that V600 mutations have worse prognosis as compared to non V600 mutations show poor treatment responses.[24,25] Since *BRAF* mutations have been reported to be independent molecular variable that defines poor survival, the clinical management of CRC patients may be directly affected by *BRAF* mutational status.[26,27] Furthermore, *BRAF* mutant cancers have been reported to be unresponsive to anti-EGRF therapy.[14] There is no reported data regarding *BRAF* mutational status in Pakistani population. Moreover, our search of ClinicalTrials.gov database did not reveal any registered clinical trials based on BRAF mutations in Pakistan (Figure-1b).

There is no defined regime which is proven to treat all CRC patients with same efficacy . However; efforts to add to existing body of information will eventually result in identifying actionable gene targets and novel therapies. Both *KRAS* and *BRAF* have been found to be associated with biological pathways that converge at MAPK signalling. It has been reported that ERK activation can result in unchecked proliferation of intestinal cells, while negative feedback to ERK is associated with drug resistance in CRC. The prolonged ERK activation events that are associated with *KRAS* and *BRAF* (p=0.016), therefore, facilitate colorectal carcinogenesis.[28] Literature supports our finding that p38 activates MEK cascade signalling via RIT and RIN (p=0.01) and that ARMS mediated activation triggers MAPK cascade (p=0.01) increasing vulnerability to CRC.[27] MAPK pathway activation also results from phosphorylation of Frs2 (p=0.01) and from Trk activation (p=0.047).[29,30] Literature shows that Trk and MEK inhibition together can regress cancer progression.[30] The identified pathways play a critical role in signal transduction from activated receptors to their downstream effecter proteins and can trigger unchecked proliferation of intestinal cells. Combination therapies targeting *KRAS*, *BRAF* and the associated biological pathways can improve patient prognosis. The presence of altered kinases in CRC have clinical implications and show great potential as predictive biomarkers for the efficacy of conventional and targeted treatments, deserving further research

CONCLUSION

Our study highlights the significant soamtic mutations associated with CRC. Targeting several collateral pathways simultaneously may be further explored to improve colorectal cancer therapeutics. Based on lack of comprehensive database of our population and a huge gap in local research, studies focused on molecular profiling of our genetically

distinct population are warranted. With Identification of our population specific mutations, we might be able to stratify subpopulations of CRC to better predict outcome and assign therapies.

AUTHOR CONTRIBUTION

SH, AK and TM conceived the project, designed it and retrieved data for this research. UB contributed to statistical analysis. All authors contributed to manuscript writing and editing .

Data Availability

Following links to online reporsitories may be used to access the data underlying the findings of our study

https://cancer.sanger.ac.uk/cosmic

http://www.internationalgenome.org/

https://clinicaltrials.gov/

https://www.ncbi.nlm.nih.gov/clinvar/

Data analyzed using Functional Enrichment tool can be accessed using the following link

http://www.funrich.org

Conflicts of Interest:

The authors declare no conflict of interest.

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Supplementary Material:

Supplementary sheet 1 shows the top 20 genes associated with colorectal adenocarcinoma as reported by COSMIC database while Supplememtary Sheet 2 shows details of PPI network of selected genes (*KRAS* and *BRAF*). Supplemetary sheet 3 contains names of suggested reviewers.

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