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Determination of the frequency and distribution of APC, PIK3CA, and SMAD4 gene mutations in Ugandan patients with colorectal cancer

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Abstract

Uganda is a developing low-income country with a low incidence of colorectal cancer, which is steadily increasing. Ugandan colorectal cancer (CRC) patients are young and present with advanced-stage disease. In our population, there is a scarcity of genetic oncological studies, therefore, we investigated the mutational status of CRC tissues, focusing in particular on the adenomatous polyposis coli (APC), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), and SMAD4 genes. Our objective was to determine whether there were any differences between other populations and Ugandan patients. We performed next-generation sequencing on the extracted DNA from formalin-fixed paraffin-embedded adenocarcinoma samples from 127 patients (mean (SD) age: 54.9 (16.0) years; male:female sex ratio: 1.2:1). Most tumours were located in the rectum 56 (44.1%), 14 (11%) tumours were high grade, and 96 (75.6%) were moderate grade CRC. Stage III + IV CRC tumours were found in 109 (85.8%) patients. We identified 48 variants of APC, including 9 novel APC mutations that were all pathogenic or deleterious. For PIK3CA, we found 19 variants, of which 9 were deleterious or pathogenic. Four PIK3CA novel pathogenic or deleterious variants were included (c.1397C > G, c.2399_2400insA, c.2621G > C, c.2632C > G). Three SMAD4 variants were reported, including two pathogenic or deleterious variants (c.1268G > T, c.556dupC) and one tolerant (c.563A > C) variant. One novel SMAD4 deleterious mutation (c.1268G > T) was reported. In conclusion, we provide clinicopathological information and new genetic variation data pertinent to CRC in Uganda.

Keywords APC, PIK3CA, SMAD4, Variants, Mutation, Colorectal cancer, Genetics, Africa, Uganda

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Introduction

Globally, in 2020, CRC was ranked as the third most common cancer-related mortality, accounting for 9% of cancer deaths and 10% of cancer incidence [1, 2]. In the scientific literature, cancer in Africa with respect to cancer genetics is generally underreported and underinvestigated [3–7]. However, despite the paucity of colorectal cancer research, data shows that Ugandan CRC patients are younger than those in other parts of the world [8, 9].

In Uganda, as in many Sub-Saharan African countries, many patients present with advanced-stage CRC with poor overall survival, and a high number of patients are lost to follow-up [9–11]. These effects may be due to the unavailability of national screening programs, limited infrastructure, limited healthcare personnel, and lack of awareness of colorectal cancer among the Ugandan population [8, 9]. The resulting late stage has an impact on treatment and prognosis.

There has been a gradual steady increase in CRC in the Ugandan population over the last two decades. This period tends to coincide with the emergence of specialized care and an improvement in CRC diagnostic capacity. The increase in the number of trained surgeons working in remote areas of the country may also explain the steady increase in the incidence of CRC in Uganda. The development of CRC is dependent on important processes that involve defective cell regulation and gene mutation [12].

There are differences in the molecular characteristics and genetic background that have been reported between Africans and other races [13, 14]. The role of genetic or hereditary factors in CRC has been estimated to be 35–40% [15–17]. Differences in somatic mutations and microsatellite stability status with CRC have been documented between different populations [13, 16]. Studies have shown that the adenomatous polyposis (APC) gene, which is a driver gene in CRC, is more altered in African Americans [15–17].

APC is a key gatekeeper gene involved in CRC development and is a multifunctional tumour suppressor gene [17–19]. APC mutations together with TP53, BRAF, and KRAS may predict the outcome of CRC; therefore, APC may play an important prognostic role in CRC [19].

An accumulation of mutations in NRAS, KRAS, BRAF, and PIK3CA activate multiple signaling pathways, including PIK3-PTEN-AKT and RAS-RAF-MAPK, which regulate cell motility, apoptosis, angiogenesis, and cell proliferation in CRC [20–22]. In colorectal carcinogenesis, an important role is played by the phosphatidylinositol 3-kinase signaling pathway [23]. In the West, 15–20% of colorectal cancers have mutations in the PIK3CA gene. This gene encodes phosphatidylinositol-4,5-bisphosphonate 3-kinase, catalytic subunit alpha polypeptide [24–30]. Inhibition of apoptosis in colon cancer cells is due to upregulation of the PI3K signaling pathway, which stimulates PTGS2 activity and prostaglandin E2 synthesis [31]. By blocking the PI3K pathway, aspirin may induce apoptosis and suppress cancer cell growth [32]. Recent evidence has shown that compared to patients with wild-type PIK3CA colon cancer, the use of aspirin among patients with mutated-PIK3CA is associated with better survival [33]. Therefore, this finding suggests that PIK3CA mutation is a predictive molecular biomarker for adjuvant therapy with aspirin [33].

One of the most common genes mutated in colorectal cancer is SMAD4, with the Cancer Genome Atlas database revealing a mutation frequency of 10%. The most commonly destroyed gene in colorectal cancer among the family of SMAD genes is SMAD4, which is a tumour suppressor gene, located on chromosome 18q21 [34]. In response to TGF-β (transforming growth factor beta) signal transduction the SMAD4 gene encodes signal transduction proteins that are activated and phosphorylated by transmembrane serine-threonine receptor kinases. In combination with other activated SMAD proteins, the product of this gene forms heteromeric complexes and homomeric complexes by activating TGF-β receptors, which regulate the transcription of target genes after accumulating in the nucleus [35]. Juvenile polyposis syndrome, hereditary hemorrhagic telangiectasia, and pancreatic cancer result from deletions or mutations in the SMAD4 gene [36–38]. The frequency of the SMAD4 gene mutation in Ugandan patients remains unknown.

Many studies in the past two decades have shown that SMAD4 can promote tumour progression caused by other genes but cannot cause tumorigenesis by itself [36, 37]. In CRC the role of SMAD4 is similar to that found in pancreatic cancer. During the 1999–2020 period, several studies have shown that the prevalence of SMAD4 mutations is between 5% and 24.2% [38–43]. A meta-analysis by Fang T et al., showed that SMAD4 mutations may be associated with aggressive clinicopathological characteristics, including lymph node metastasis, TNM stage, tumour size and mucinous differentiation, which may predict a poor prognosis [44].

Studies have shown that compared to CRC patients from Western countries, those from China and the Arab world have differences in the genetic profiles of APC, NRAS, BRAF, KRAS and PIK3CA at mutation hotspots [45]. In Ugandan patients, the rate of these mutations in CRC is not well defined.

We conducted this study to evaluate the mutational status in colorectal cancer tissues due to the young age of presentation, the steady increase in incidence observed in the Ugandan population and the paucity of oncogenetic colorectal studies in this population. We focused on frequently mutated CRC genes particularly APC, PIK3CA

and SMAD4. The aim of this study was to determine any differences in these mutated genes between other populations and the Ugandan population. In this paper, we describe the frequency of genetic mutations in the APC, PIK3CA and SMAD4 genes, including novel mutations in CRC tissues from Ugandan patients.

Methodology

From the 16th of September 2019 to the 16th of September 2021, we recruited prospectively, consecutive participants attending the Surgical Departments of Masaka Regional Referral Hospital, Mulago National Referral Hospital, Uganda Martyrs' Hospital Lubaga and Mengo Hospital. From 1st January 2008 to 15th September 2021, retrospective CRC FFPE blocks were obtained from the archives of the Department of Pathology, School of Biomedical Sciences, College of Health Sciences, Makerere University. The histopathologic diagnosis was confirmed as colorectal adenocarcinoma by one consultant pathologist at the Department of Pathology, School of Biomedical Sciences, College of Health Sciences, Makerere University, and another consultant pathologist at the Institute of Genetics and Cancer at the University of Edinburgh.

Cases with a histologically proven diagnosis of colorectal adenocarcinoma were considered for inclusion. Cases with recurrent colorectal cancer and poor-quality FFPE tissue samples were excluded. Poor quality FFPE tissue samples included those having a low concentration of extracted DNA, poor quality DNA, or insufficient tissue for the extraction of DNA. All cases meeting the selection criteria during the study period were included.

The stage of the CRC was radiological, obtained from the radiology reports in the participants' medical case files. The radiological TNM staging (8th edition) was used to stage all the colorectal tumours. The CRC cases were graded using a three-tier grading system as follows: well-differentiated CRC (G1) with > 95% glandular formation; moderately differentiated CRC (G2) with 50–95% glandular formation; and poorly differentiated CRC (G3) with < 50% glandular formation (Fletcher CDM et al., 2019). Data were obtained from the clinical case files for demographics, radiological stage, and topography of the colorectal tumour.

Extraction of DNA

From formalin-fixed paraffin-embedded (FFPE) tissue blocks that contained at least 50% tumour, DNA was extracted. FFPE tissue blocks with DNA degradation were excluded The QIAamp DNA FFPE Advanced UNG kits (Qiagen GmbH, Hilden, Germany) were used for DNA extraction following the recommendations of the manufacturer. A Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, CO, USA) was used to measure the concentration and quality of the extracted DNA. The quality of each sample was checked on the Qubit to make sure it fell within 100-250 ng of DNA required for the DNA protocol. In order to prevent degradation it was stored at -20^{0}C. All the 127 CRC DNA samples passed the quality check.

Library preparation and NGS sequencing

Library preparation was completed following the QIAseq targeted DNA Pro kit for Illumina (Qiagen GmbH, Hilden, Germany) along with a Qiagen custom design panel (QIAseq DNA panel catalogue identifier: CPHS-43072Z-1294) [46]. The custom panel represented 56 genes with a total of 1,294 primer probes. It is designed to enrich selected genes and regions using 100 to 250 ng FFPE DNA. A single controlled multienzyme reaction was responsible for the fragmentation of the DNA samples, end repair, and A-tailing. A sequencing platform-specific adapter containing UMI prepared DNA fragments was used which ligated their 5' ends. To generate more FFPE DNA molecules for library construction, a repair step was carried out. The repaired FFPE DNA was placed directly into the fragmentation reaction in the same tube. An adaptor containing a 12-base fully random sequence (ie., UMI) was used to ligate the fragmented DNA. A unique sequence was used for each DNA molecule in the sample. Following the UMI assignment, target enrichment was performed to ensure that DNA molecules with UMIs in the sequenced library were sufficiently enriched. Several cycles of targeted PCR using one universal primer complementary to the adaptor and one region-specific primer were subjected to ligated DNA molecules for enrichment. Amplification of the library and addition of platform-specific adaptor sequences and sample indices were carried out using universal PCR.

An enzymatic reaction was used for cleanup after ligation and target enrichment PCRs. Following enzymatic cleanups, more consistent library construction was achieved, as there were no highly variable bead cleanups following ligation and target enrichment PCRs [47] (Fig. 1).

Two unique indices were assigned to each sample o overcome errors due to image analysis, demultiplexing sequencing error, and oligo synthesis contamination to reduce any real misassignment to incorrect samples. The library pool was sequenced on the Illumina MiSeq platform using a dual indexed paired-end sequencing program of 2 × 149-bp reads.

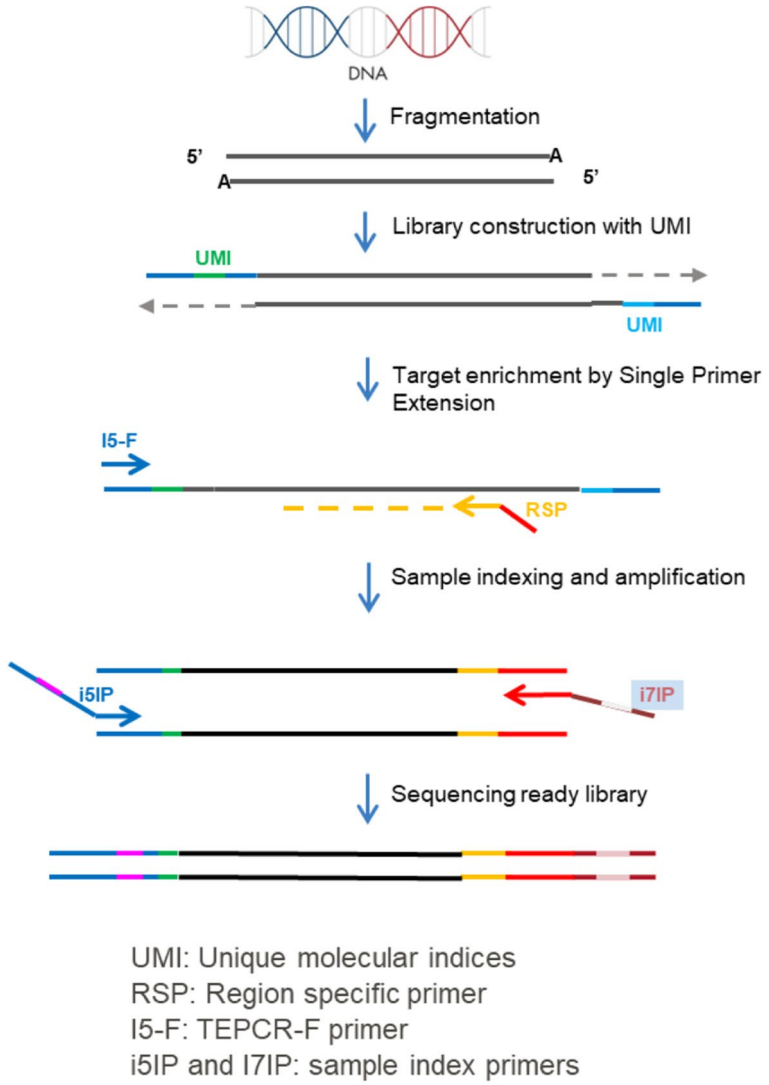

Fig. 1 QIAseq library preparation workflow (adapted from QIAseq Targeted DNA Pro Handbook Page 11)

Data analysis

The quality of the raw FASTQ files was first assessed using FastQC and MultiQC software [48–50], which generated HTML quality reports. Bases with a Phred quality score below 25 and adapter sequences were trimmed using Trim Galore [51].

The processed reads were aligned to the human genome reference version 38 (hg38) using BWA-MEM [52], generating the alignment files. Variant discovery was performed using the GATK4 (Genome Analysis Tool Kit version 4) pipeline following the best practices guidelines [53], employing the HaplotypeCaller option. Variants were filtered for downstream analysis based on an overall read depth greater than 20X and a variant allele depth of at least 10X.

The resultant variants were annotated using ANNOVAR (Wang et al., 2010). Variants of uncertain significance (VUS) according to ClinVar [54] were subjected to further analysis using nine variant effect prioritization tools: SIFT [55], LRT [56], MutationTaster [57], Mutation Assessor (Reva et al., 2011), FATHMM [50], PROVEAN [50], ClinPred [48], MutPred [52], and MetaSVM [58]. A variant was classified as deleterious (D) if at least five out of these nine tools predicted it to have a damaging effect. This threshold was chosen based on the consensus approach used in previous studies to minimize false positives and ensure a high confidence in pathogenicity classification [53, 55, 59]. Variants predicted to be damaging by fewer than five tools were considered tolerated (T).

To determine the novelty of mutations, we screened their absence in major population and mutation databases: COSMIC [57], 1000 Genomes [60], dbSNP [51, 54, 57, 58, 60–63], ExAC [64], GnomAD [62], ClinVar, Varsome [63], and Mastermind. This approach was to ensure the robustness and reliability of our accurate interpretation and potential clinical application [51–55, 59, 63, 64].

Results

Clinicopathological characteristics of CRC participants

We analyzed data from 127 patients, with a mean (SD) age of 54.9 (16.0) years. There were 69 (54.3%) males and 58 (45.7%) females with CRC. AJCC early-stage (I + II) CRC constituted 18 (14.2%) patients, while late-stage (III + IV) CRC was found in 109 (85.8%) patients. There were 17 (13.4%) patients with well-differentiated CRC, 96 (75.6%) with moderately differentiated CRC and 14 (11%) poorly differentiated CRC. The majority of tumours were found in the rectum, 56 (44.1%), followed by sigmoid colon tumours 20 (15.8%).

Characteristics of genetic APC variants

We found 48 different genetic variants of the APC gene (Table 1; Fig. 1). These included 9 novel genetic mutations of which four were pathogenic and five were deleterious variants (Table 2). The global and African frequencies for the APC Ugandan variants were found in the 1000 Genomes Project and ExAC population databases (Table 3). There were 39 mutations that were previously reported, and 5 single-nucleotide polymorphisms (SNPs): rs2229992, rs351771, rs41115, rs459552 and rs465899. A minor allele frequency (MAP) of > 50% in major databases was found with these SNPs. Excluding these single nucleotide polymorphisms, 43 (43/127; 33.9%) cases had at least one mutation. The most common deleterious/pathogenic variants included rs777568434 (c.3482A > G; p.Y1161C) (57/127; 44.9%), which was classified as a deleterious variant and (c.4933_4934insC; p.E1645Afs*6), which was classified as a pathogenic variant. The most common benign APC variant included c.3485G > C (p.S1162T). Recurrent mutations included (c.1480_1481insC; p.D494Afs*25) (4/127), (c.2127G > C; p. R709S) (35/127), rs78429131 (c.31 T > G) (2/127), (c.2128A > T; p. N710Y) (4/127), rs777568434 (c.3482A > G; p. Y1161C) (57/127), (c.3485G > C; p. Y1161C) (57/127), (c.3485G > C; p. S1162T) (38/127), (c.3592G > C; p. E1198Q) (8/127), rs1554085294 (c.3594G > A; p. E1198E) (8/127), (c.379 T > A; p. S127T) (4/127), (c.4253G > A; p. S1418N) (4/127), (c.4933_4934insC; p. E1645Afs*6) (33/127), (c.5388_5389insC; p. N1797Qfs*5) (3/127), (c.5498G > C; p. G1833A) (3/127), (c.5499A > T; p. G1833G), (c.5727 T > G; p. L1909L) (4/127), rs1186128913 (c.5728C > T; p. Q1910X) (4/127), rs1561605162 (c.6067G > C; p. E2023Q) (5/127), (c.6630G > T; p. R2210S) (6/127), (c.6961C > A, p. P2321T) (32/127), (c.7304G > C; p. R2435T) (9/127), (c.7308A > T; p. R2436S) (9/127), (c.7420_7421insA; p. S2474fs*19) (6/127), rs1455101572 (c.7423C > G; p. L2475V) (6/127), (c.7654 T > A; p. S2552T) (2/127).

Frameshift insertion mutations, which were all pathogenic, were found in 47/127 (37%) cases. Stop gain mutations were found in 7/127 (5.5%) cases and all these cases were pathogenic. There were 128 mutations of uncertain clinical significance, and using in-silico bioinformatics tools, 95/127 (74.8%) mutations were deleterious and 33/127 (25.9%) mutations were benign.

Characteristics of Genetic PIK3CA variants

There were 19 different genetic mutation variants of the PIK3CA gene (Table 4; Fig. 2), which were detected in 51 CRC patients. The novel mutations included four variants (Table 5). The global and African frequencies for the PIK3CA Ugandan variants were found in the 1000 Genomes Project and ExAC population databases (Table 6). The most frequently reported deleterious mutations were (c.124 T > G; p.L42V) (25/127; 19.7%) followed by (c.2621G > C; p. S874T) (20/127; 15.7%). The most frequently reported pathogenic mutations were (c.2399_2400insA; p.801Lfs*2) (7/127; 5.5%) followed by (c.2906_2907insC; p. Q969Hfs*10) (6/127; 4.7%) and (c.2908G > A; p. E970K) (6/127; 4.7%). The most frequent tolerant mutation was rs200031978 (c.1535G > T; p.G512V) (45/127; 35.4%). This mutation has been reported to have a minor allele frequency of 0.0000 in African populations in the ExAC population database and GnomAD-genome + exome population databases. The ExAC population database found an MAF of 0.001 in South Asia and an MAF of 6.58×10^{-6} globally in the GnomAD-genome and 8.04×10^{-6} globally in the GnomAD-exome population database.

There was a single nucleotide polymorphism (SNP) variant, rs2230461 (c.1173A > G; p. I391M), which was reported in one case.

Characteristics of Genetic SMAD4 variants

There was at least one SMAD4 genetic variant identified in 68 patients (Table 7; Fig. 2). One novel SMAD4 mutation (c.1268G > T; pG423V), which was a missense variant, had clinical significance and was identified as deleterious. There were a total of 68 cases with the (c.556dupC; p. S187Kfs*2) pathogenic variant. There were 5 cases with the novel deleterious variant (c.1268G > T; p. G423V) (Table 8). The global and African frequencies for the SMAD4 Ugandan variants were found in the 1000 Genomes Project and ExAC population databases (Table 9).

Table 1 Summary of APC variants identified in Ugandan CRC patients: each variant is characterized by its mutation type, dbSNP ID, HGVS (Human Genome Variation Society) notation for coding (c.) and protein (p.) changes, clinical significance, and the number of cases with each mutation

Variant No	Type of mutation	dbSNPID	HGVS.c	HGVS.p	Clinical significance	No. of cases with mutation
1	Synonymous	rs142720069	c.120G > A	p.E40E	Benign	1
2	Synonymous	rs2229992	c.1404 T > C	p.Y468Y	Benign	2
3	Frameshift insertion	N/A	c.1480_1481insC	p.D494Afs25	Pathogenic	4
4	Synonymous	rs351771	c.1581G > A	p.A527A	Benign	5
5	Synonymous	rs77921116	c.1641A > G	p.E547E	Benign	1
6	Missense	N/A	c.2127G > C	p.R709S	Benign	35
7	Missense	N/A	c.2128A > T	p.N710Y	Deleterious	4
8	Missense	N/A	c.2284A > T	p.S762C	Benign	1
9	Missense	rs763546422	c.2460A > T	p.R820S	Benign	1
10	UTR5	rs78429131	c.31 T > G	-	Benign	2
11	Missense	rs777568434	c.3482A > G	p.Y1161C	Deleterious	57
12	Missense	N/A	c.3485G > C	p.S1162T	Benign	38
13	Missense	N/A	c.3592G > C	p.E1198Q	Benign	8
14	Synonymous	rs1554085294	c.3594G > A	p.E1198E	Benign	8
15	Synonymous	rs74380081	c.3678A > G	p.Q1226Q	Benign	1
16	Missense	N/A	c.379 T > A	p.S127T	Benign	4
17	Missense	N/A	c.4253G > A	p.S1418N	Deleterious	4
18	Synonymous	rs41115	c.4425G > A	p.T1475T	Benign	8
19	Frameshift insertion	N/A	c.4933_4934insC	p.E1645Afs6	Pathogenic	33
20	Synonymous	rs42427	c.4980G > A	p.G1660G	Benign	1
21	Frameshift insertion	N/A	c.5161_5162insC	p.K1721Tfs30	Pathogenic	1
22	Frameshift insertion	N/A	c.5388_5389insC	p.N1797Qfs5	Pathogenic	3
23	Missense	rs459552	c.5411 T > A	p.V1804D	Benign	21
24	N/A	N/A	c.5498G > A	p.G1833E	Tolerant	1
25	Missense	N/A	c.5498G > C	p.G1833A	Deleterious	3
26	Synonymous	N/A	c.5499A > T	p.G1833G	Benign	35
27	Synonymous	N/A	c.5502 T > G	p.T1834T	Benign	1
28	Missense	N/A	c.5504C > A	p.P1835H	Deleterious	1
29	Synonymous	N/A	c.5505 T > A	p.P1835P	Benign	1
30	Synonymous	N/A	c.5727 T > G	p.L1909L	Benign	4
31	Stop gained	rs1186128913	c.5728C > T	p.Q1910X	Pathogenic	4
32	Synonymous	rs465899	c.5826G > A	p.P1942P	Benign	10
33	Missense	rs1561605162	c.6067G > C	p.E2023Q	Deleterious	5
34	Missense	N/A	c.6630G > T	p.R2210S	Deleterious	6
35	Stop gained	N/A	c.6633delA	p.M2212	Pathogenic	1
36	Missense	N/A	c.6636G > A	p.M2212I	Deleterious	1
37	Missense	N/A	c.6637A > C	p.T2213L	Deleterious	1
38	Missense	N/A	c.6638 T > C	p.I2213T	Deleterious	1
39	Stop gained	rs587781392	c.667C > T	p.R223X	Pathogenic	1
40	Missense	N/A	c.6961C > A	p.P2321T	Tolerant	32
41	Missense	N/A	c.7304G > C	p.R2435T	Benign	9
42	Missense	N/A	c.7308A > T	p.R2436S	Deleterious	9
43	Frameshift insertion	N/A	c.7420_7421insA	p.S2474fs19	Pathogenic	6
44	Missense	rs1455101572	c.7423C > G	p.L2475V	Benign	6
45	Synonymous	rs35043160	c.7650A > G	p.G2550G	Benign	1
46	Missense	N/A	c.7652G > C	p.S2551T	Deleterious	1
47	Missense	N/A	c.7654 T > A	p.S2552T	Deleterious	2
48	Stop gain	Rs137854568	c.850C > T	p.R284X	Pathogenic	1

Table 2 Novel APC variants identified in Ugandan colorectal cancer patients

Variant No	HGVSc	HGVSp	Type of mutation	Clinical significance
1	c.4253G > A	p.S1418N	Missense	Deleterious
2	c.4933_4934insC	p.E1645Afs6	Framehsift insertion	Pathogenic
3	c.5161_5162insC	p.K172Tfs30	Frameshift insertion	Pathogenic
4	c.5498G > C	p.G1833A	Missense	Deleterious
5	c.5504C > A	p.P1835H	Missense	Deleterious
6	c.6630G > T	p.R2210S	Missense	Deleterious
7	c.6633delA	p.M2212	Stop gained	Pathogenic
8	c.6638 T > C	p.I12213T	Missense	Deleterious
9	c.7420_7421insA	p.S2474Yfs19	Framehsift insertion	Pathogenic

Discussion

In the last two decades up to 2019, to the knowledge of the authors, only two peer-reviewed published papers appeared in scientific journals reporting on colorectal cancer in Uganda [65, 66]. In the Ugandan population, CRC is underinvestigated, especially, the molecular genetic profile of the tumour. In his study, we found that CRC was more commonly distributed among male than female patients (54.3% versus 45.7%). This is consistent with global data that have reported a male predominance in the sex distribution of colorectal cancer [1]. However in Rwanda, studies have shown a higher female predominance of CRC: Manirakiza F et al., 2023; 63%, Chiorean EG et al., 2020; 52.2% and Uwamariya D et al., 2022; 52.5% [67–69]. In Rwanda the sample size in the study by Manikariza F et al., was small and hence the finding on gender disparity may not reflect the entire population [67]. Colorectal cancer tends to affect males more commonly in Uganda and globally as risk factors such as smoking and alcohol consumption tend to be more commonly associated with the male rather than female gender [8].

Most patients (85.8%) in our study population presented with late-stage disease [67–69]. This may be due to lack of screening but also due to patients presenting late to hospital with signs and symptoms of large bowel obstruction rather than with early symptoms.

In this study, most tumours were located in the rectum (44.1%), followed by sigmoid colon tumours. This may be due to rectal tumours being more symptomatic than colon tumours and presenting with bleeding, blood in the stool and tenesmus. Therefore, compared to colon tumours, patients presenting with rectal tumours are relatively more likely to seek surgical care. Additionally, a national screening programme is not available in Uganda and generally in Sub-Saharan Africa [70]. Therefore, many colon cancer patients present with late-stage disease, with signs and symptoms of large bowel obstruction. Hence many colon tumours undergo resection without undergoing a colonoscopy in Uganda.

There was missing information that was not recorded regarding family history in the prospectively recruited patients (30/82; 36%). There were 42/82 (51.2%) patients who did not report any family history of CRC. Only 8/82 (9.8%) patients reported having a family history of CRC. Therefore, less than 10% of patients reported having a family history of CRC. This may not necessarily mean that CRC tumours were not present, as the data regarding family history were self-reported, and in the past, there were limitations in the diagnosis of CRC and treatment. Cancer registration in the past was also limited in Uganda [70, 71]. Therefore, one should interpret with caution any data regarding family history of CRC.

Mutations in the APC gene

In the development of CRC, the APC gene is a key gatekeeper gene (Schell MJ et al., 2016; Augustus GJ et al., 2018). A key factor in familial adenomatous polyposis syndrome is germline mutation in APC. This syndrome is rare in African populations, with only a few case reports in the literature [72–78]. In more than 50% of cases, mutations in the APC gene occur in CRC [79]. More than 60% of these APC mutations are located in the mutation cluster region (MCR) [79, 80]. In the MCR region, 1339–1436 codons gave the highest mutation frequency, while 1260–1359 codons gave the lowest mutation frequency in a study by Wang Y et al. [81]. The APC gene is mutated early in colorectal carcinogenesis, and this mutation has been detected in colorectal adenomas. Early detection of APC mutations helps in establishing an early diagnosis of CRC.

In our study, we did not limit our genetic assessment only to the mutation cluster region in comparison to Manirakiza F et al., 2023 from Rwanda [67]. We detected over 60.6% pathogenic APC mutations in our patient population. There is a higher minimal allele frequency of

Table 3 APC variants in Ugandan CRC patients compared with Minor Allele Frequency (MAF) in Global and African Genome Databases of 1000 Genomes Project and ExAC (Exome Aggregation Consortium) databases

Variant No	HGVS.c	1000 Genomes Project (MAF)		ExAC Population (MAF)	
		Global	African	Global	African
1	c.120G > A	1E-03	0.003	7E-04	0.006
2	c.1404 T > C	0.51	0.162	0.578	0.205
3	c.1480_1481insC	N/A	N/A	N/A	N/A
4	c.1581G > A	0.666	0.517	0.648	0.537
5	c.1641A > G	0.014	0.054	0.004	0.038
6	c.2127G > C	N/A	N/A	N/A	N/A
7	c.2128A > T	N/A	N/A	N/A	N/A
8	c.2284A > T	N/A	N/A	N/A	N/A
9	c.2460A > T	N/A	N/A	N/A	N/A
10	c.31 T > G	0.077	0.095	0.111	0.08
11	c.3482A > G	N/A	N/A	N/A	0
12	c.34856G > C	N/A	N/A	N/A	N/A
13	c.3592G > C	N/A	N/A	N/A	N/A
14	c.3594G > A	N/A	N/A	N/A	N/A
15	c.3678A > G	0.014	0.054	0.004	0.038
16	c.379 T > A	N/A	N/A	N/A	N/A
17	c.4253G > A	N/A	N/A	N/A	N/A
18	c.4425G > A	0.666	0.517	0.649	0.537
19	c.4933_4934insC	N/A	N/A	N/A	N/A
20	c.4980G > A	0.667	0.519	0.651	0.542
21	c.5161_5162insC	N/A	N/A	N/A	N/A
22	c.5388_5389insC	N/A	N/A	N/A	N/A
23	c.5411 T > A	0.865	0.991	0.798	0.957
24	c.5498G > A	N/A	N/A	N/A	N/A
25	c.5498G > C	N/A	N/A	N/A	N/A
26	c.5499A > T	N/A	N/A	N/A	N/A
27	c.5502 T > G	N/A	N/A	N/A	N/A
28	c.5504C > A	N/A	N/A	N/A	N/A
29	c.5505 T > A	N/A	N/A	N/A	N/A
30	c.5727 T > G	N/A	N/A	N/A	N/A
31	c.5728C > T	N/A	N/A	N/A	N/A
32	c.5826G > A	0.667	0.521	0.649	0.541
33	c.6067G > C	N/A	N/A	N/A	N/A
34	c.6630G > T	N/A	N/A	N/A	N/A
35	c.6633delA	N/A	N/A	N/A	N/A
36	c.6636G > A	N/A	N/A	N/A	N/A
37	c.6637A > C	N/A	N/A	N/A	N/A
38	c.6638 T > C	N/A	N/A	N/A	N/A
39	c.667C > T	N/A	N/A	N/A	N/A
40	c.6961C > A	N/A	N/A	N/A	N/A
41	c.7304G > C	N/A	N/A	N/A	N/A
42	c.7308A > T	N/A	N/A	N/A	N/A
43	c.7420_7421insA	N/A	N/A	N/A	N/A
44	c.7423C > G	N/A	N/A	N/A	N/A
45	c.7650A > G	0.035	0.128	0.009	0.095
46	c.7652G > C	N/A	N/A	N/A	N/A
47	c.7654 T > A	N/A	N/A	N/A	N/A
48	c.850C > T	N/A	N/A	N/A	N/A

c.1404 T > C mutation in the APC gene globally compared to the African population. The minimal allele frequencies of the c.4425G > A, c.5411 T > A and c.5826G > A mutations in the APC gene in Uganda are comparable in the African and global populations.

Nine new mutations, i.e., c.4253G > A, c.4933_4934insC, c.5161_5162insC, c.5498G > C, c.5504C > A, c.6630G > T, c.6633delA, c.6638 T > C and c.7420_7421insA, have to our knowledge not been reported in major genetic variation databases, including the 1,000 Genomes Project, ExAC population, gnomAD genome population, gnomAD exome population databases and in the literature, including PubMed. Four of these novel APC mutations were missense, three novel APC mutations were frameshift insertion mutations, and one novel APC mutation was a stop gain mutation. Missense mutations may affect DNA-transcription factors, resulting in alterations in the protein expression and disrupting the normal cell cycle. The frameshift mutations may produce a truncated protein following a premature termination codon. Four of these novel mutations were of uncertain significance; however, using in silico bioinformatics tools, these genetic variants were predicted to be deleterious. The c.3482A > G mutation was found to be deleterious and was quite predominant in our study with fifty-seven mutations recorded however this mutation has not been recorded in other African countries in the ExAC Population database. Global data regarding this mutation is also currently not available in the ExAC Population database and 1,000 Genomes Project.

PIK3CA gene mutations

Previous studies of non-Hispanic white CRC individuals have shown that the prevalence of somatic PIK3CA mutations among Asian American and African American individuals with CRC is 10–20% [82, 24, 27, 83–85]. Another study by Kang et al., reported the prevalence of PIK3CA mutations in African Americans and found no significant difference in the prevalence of PIK3CA somatic mutations between white CRC cases and African American CRC cases. In East Asian and Chinese CRC

Table 4 Summary of PIK3CA variants identified in Ugandan CRC patients: each variant is characterized by its mutation type, dbSNP ID, HGVS (Human Genome Variation Society) notation for coding (c.) and protein (p.) changes, clinical significance, and the number of cases with each mutation

Variant No	Type of mutation	dbSNPID	HGVS.c	HGVS.p	Clinical significance	No. of cases with mutation
1	Missesnse	rs2230461	c.1173A > G	p.I391M	Benign	1
2	Synonymous	N/A	c.123A > T	p.T41T	Benign	25
3	Missense	N/A	c.124 T > G	p.L42V	Deleterious	25
4	Missense	N/A	c.127A > C	p.I43L	Benign	25
5	Missense	N/A	c.1395 T > A	p.N465K	Deleterious	3
6	Missense	N/A	c.1397C > G	p.P466R	Deleterious	3
7	Synonymous	N/A	c.1398A > C	p.P466P	Benign	3
8	Missense	N/A	c.1403A > C	p.K468T	Tolerant	3
9	Missense	N/A	c.1404A > T	p.K468N	Tolerant	3
10	Missense	rs200031978	c.1535G > T	p.G512V	Tolerant	45
11	Synonymous	rs116336243	c.2181A > T	p.T727T	Benign	1
12	Frameshift insertion	N/A	c.2399_2400insA	p.F801Lfs2	Pathogenic	7
13	Missense	rs1261983174	c.2402 T > G	p.F801C	Deleterious	7
14	Missense	N/A	c.2621G > C	p.S874T	Deleterious	20
15	Missense	N/A	c.2632C > G	p.H878D	Deleterious	1
16	Frameshift insertion	N/A	c.2906_2907insC	p.Q969Hfs10	Pathogenic	6
17	Missense	N/A	c.2913C > T	p.E970K	Pathogenic	6
18	Synonymous	N/A	c.2913C > T	p.C971C	Benign	4
19	Synonymous	N/A	c.2916A > T	p.T972T	Benign	1

patient populations the prevalence of PIK3CA mutations has been reported to be between 7.5% and 12.3% [86–88].

Our study showed that the prevalence of pathogenic PIK3CA mutations in Ugandan patients is 22%, which is in keeping with American patients. The mucinous CRC histological subtype is frequently associated with the presence of PIK3CA mutations [89, 85]. In advanced tumours RAS and BRAF mutations frequently coexist with PIK3CA mutations [90].

The c.2908G > A; pE940K pathogenic PIK3CA variant has been reported in the COSMIC database in five articles that have been published mainly from the USA in The Cancer Genome Atlas Network. Our sample cohort did not yield any PIK3CA mutations in the "hotspots" located in exon 20 (H1047) or exon 9 (E542K, E545K), which have been found in other studies [23]. Studies have shown that the "hotspots" of the 20th exon (codon 1047) and 9th exon (codons 542 and 545) are present in 80% of PIK3CA mutations [23]. Simultaneous PIK3CA mutations in both the 20th and 9th exons are rare; however, mutations in the 9th exon are found at a higher frequency [27, 83, 89]. The PIK3CA substitutions in exon 20, in the absence of RAS mutations, are a marker of inefficiency of anti-EGFR therapy and hence are associated with a poor prognosis [27, 91, 92].

The four novel PIK3CA mutation variants were all pathogenic or deleterious in our study. The novel pathogenic variant (c.2399_240insA; p.F801Lfs*2) was due to a frameshift insertion that produced a truncated protein following a premature termination codon. The other three novel deleterious PIK3CA variants were caused by a missense mutation. These three deleterious mutations and one pathogenic mutation were not reported in the 1,000 Genomes Project and the ExAC Population together with other global genomic databases.

There were two benign PIK3CA mutations which were more common in African rather than global populations in the ExAC population database and 1,000 Genomes project and these mutations were c.1173A > G and c.2181A > T.

A good response to treatment with aspirin is observed in individuals sssswith PIK3CA mutations together with overexpression of cyclooxygenase COX-2 [27, 93, 94]. A study by Li et al., showed an improvement in CRC overall survival with treatment with aspirin in tumours having PIK3CA mutations and PTGS2 (COX-2) expression [95]. Paleari et al., also observed a survival advantage with aspirin in patients with mutated-PIK3CA tumours with a 29% reduction in total mortality [96]. In the substrate binding channel, aspirin causes more inhibition potency for COX-1 than COX-2 and irreversibly

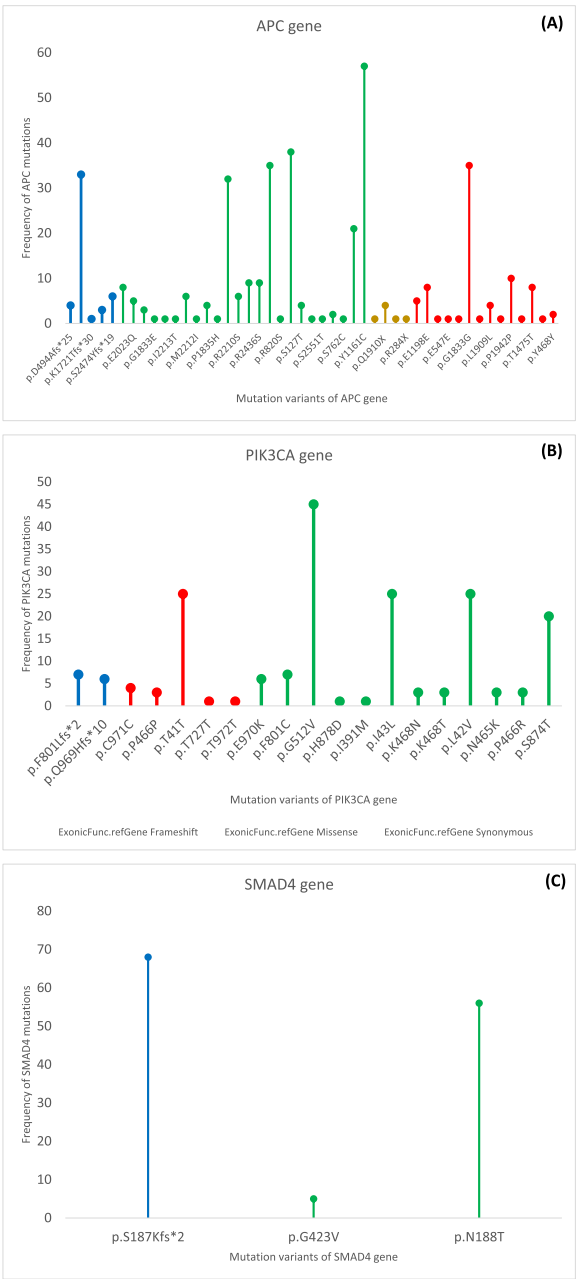

Fig. 2 Lollipop plot showing the Frequency of the different identified variants represented by their protein product and colored by their function consequence (Frameshift , Missense , Synonymous , and Stop gain). **A** APC gene variants, **B** PIK3CA gene variants and **C** SMAD4 gene variants

inactivates the enzyme [96]. The precise mechanisms of interplay between aspirin and mutations in PIK3CA are still unclear [27, 91, 97].

There are several mechanisms that are responsible for the anticancer activity of aspirin. Aspirin has been shown to downregulate the NF-kB, Akt/mTOR, PKA, β-catenin, and MAPK pathways [98, 99]. Aspirin inhibits COX-2 and activates EP1-4 prostaglandin receptors while preventing the synthesis of prostaglandin E2. EP1-4 prostaglandin receptor activation results in colonic tumorigenesis and invasion [100, 101]. In colon cancer cells, β-catenin is activated by EP2, which is part of the Wnt/β-catenin pathway, and plays a role in CRC induction, invasion and growth. Stimulation of EP2, results in the release of β-catenin from the β-catenin/GSK3β/Axin/APC complex. This release of β-catenin occurs in two ways: 1) β/γ subunits of G-protein activate Akt and PI3K, and the latter causes GSK3β phosphorylation. 2) Axin interacts with the β-γ subunits of G-protein activating Akt and PI3K [102, 103].

The MAPK pathway is activated in the prostaglandin receptor EP4 in two ways: 1) PI3K activates MAPK signaling (Wang D et al., 2011; Buchanan FG et al., 2006; Regan JW et al., 2003). EP4 has proangiogenic activity, which is mediated by protein kinase A pathways [20]. 2) Src-β-arrestin-mediated activation of EGFR is induced by EP4 [20].

SMAD4 gene mutations

The SMAD4 gene is located on chromosome 18q21. Western literature has reported loss of the SMAD4 gene locus in 30–40% of patients, with 70% of colorectal cancer cases exhibiting loss of heterozygosity on chromosome 18 [104]. The development of CRC results from loss of function of the SMAD4 gene [46]. In advanced-stage CRC, many publications have shown the role of the SMAD4 gene. In large bowel cancer, a frequent feature is loss of SMAD4 gene expression and this event is more frequent in patients with distal metastasis of CRC [105, 106]. The association between susceptibility to colorectal cancer and the occurrence of SMAD4 gene mutations is well known. Somatic mutations in the SMAD4 gene may lead to sporadic colon cancer [40].

Slattery et al., have shown that many genetic mutations in genes involving the TGF-β pathway are responsible for developing colon and rectal cancer [107]. These researchers showed a decreased risk of rectal cancer in women and an increased risk of rectal cancer in men with a mutation in the SMAD4 gene, particularly the rs10502913 variant. There were no rs10502913 variants in the SMAD4 gene reported in our Ugandan CRC patients. In our study on Ugandan CRC patients, there were 68 cases that were due to a pathogenic variant (c.556dupC) in the SMAD4 gene, and this was a frameshift insertion. This pathogenic mutation has not been reported in the 1000 Genomes Project or the ExAC Population database in global and African populations.

Table 5 Novel PIK3CA variants identified in Ugandan colorectal cancer patients

Variant No	HGVSc	HGVSp	Type of mutation	Clinical significance
1	c.1397C > G	p.P466R	Missense	Deleterious
2	c.2399_240insA	p.F801Lfs2	Frameshift insertion	Pathogenic
3	c.2621G > C	p.S874T	Missense	Deleterious
4	c.2632C > G	p.H878D	Missense	Deleterious

Table 6 PIK3CA variants in Ugandan CRC patients compared with Minor Allele Frequency (MAF) in Global and African Genome Databases of 1000 Genomes Project and ExAC (Exome Aggregation Consortium) databases

Variant No	HGVS.c	1000 Genomes Project (MAF)		ExAC Population (MAF)	
		Global	African	Global	African
1	c.1173A > G	0.088	0.224	0.065	0.209
2	c.123A > T	N/A	N/A	N/A	N/A
3	c.124 T > G	N/A	N/A	N/A	N/A
4	c.127A > C	N/A	N/A	N/A	N/A
5	c.1395 T > A	N/A	N/A	N/A	N/A
6	c.1397C > G	N/A	N/A	N/A	N/A
7	c.1398A > C	N/A	N/A	N/A	N/A
8	c.1403A > C	N/A	N/A	N/A	N/A
9	c.1404A > T	N/A	N/A	N/A	N/A
10	c.1535G > T	N/A	N/A	N/A	0
11	c.2181A > T	1E-03	0.004	3E-04	0.003
12	c.2399_2400insA	N/A	N/A	N/A	N/A
13	c.2402 T > G	N/A	N/A	N/A	N/A
14	c.2621G > C	N/A	N/A	N/A	N/A
15	c.2632C > G	N/A	N/A	N/A	N/A
16	c.2906_2907insC	N/A	N/A	N/A	N/A
17	c.2908G > A	N/A	N/A	N/A	N/A
18	c.2913C > T	N/A	N/A	N/A	N/A

In Dukes B colorectal tumours, studies have shown that allelic loss in 18q is associated with a poor prognosis [108, 109]. However, in lymph node-positive Dukes C patients the significance of these deletions is still controversial, with some studies showing no prognostic value [109–113] and other studies showing improved survival [108, 109, 114]. In our study in Uganda, our sample size was too small to evaluate the prognostic significance of the SMAD4 mutation on survival. However, other studies have shown that SMAD4 is a predictive biomarker for 5-fluorouracil (5-FU) based chemotherapy in CRC patients [115]. A poor overall survival was found following liver resection with SMAD4 mutated CRC, which was independent of RAS mutation status (Mizuno et al.). Studies in CRC patients have generally found that SMAD4 mutation was associated with a poor prognosis. This is independent of tumour grade, BRAF status and MSI status [48–50, 56, 57, 116–119].

At molecular and genetic levels CRC is a heterogenous disease. A large number of CRC cases may arise from polyps that may be cured by simple resection [51–64]. Widely accepted mechanisms of CRC development include the pure microsatellite instability pathway, the chromosomal instability pathway (CIN) which accounts for more than 80% of CRC cases and the CpG island methylation phenotype (CIMP) that leads to development of CRC [120, 121]. Over the past two decades, research has been carried out to determine the genetic mutations and molecular abnormalities associated with colorectal adenomas and CRC in order to upscale screening programmes [122–126]. This has yielded promising results in developed high-income countries with many CRC cases now being diagnosed before the onset of symptoms.

In Uganda, screening programmes are yet being conceptualized with many patients presenting with late stage CRC. The family history of colorectal cancer is often underreported in Sub-Saharan Africa. It is

Table 7 Summary of SMAD4 variants identified in Ugandan CRC patients: each variant is characterized by its mutation type, dbSNP ID, HGVS (Human Genome Variation Society) notation for coding (c.) and protein (p.) changes, clinical significance, and the number of cases with each mutation

Variant No	Type of mutation	dbSNPID	HGVS.c	HGVS.p	Clinical significance	No. of cases with mutation
1	Missense	N/A	c.1268G > T	p.G423V	Deleterious	5
2	Frameshift insertion	N/A	c.556dupC	p.S187Kfs2	Pathogenic	68
3	Missense	N/A	c.563A > C	p.N188T	Tolerant	56

Table 8 Novel SMAD4 variants in Ugandan CRC patients

Variation No	HGVSc	HGVSp	Type of mutation	Clinical significance
1	c.1268G > T	p.G423V	Missense	Deleterious

Table 9 SMAD4 variants in Ugandan CRC patients compared with Minor Allele Frequency (MAF) in Global and African Genome Databases of 1000 Genomes Project and ExAC (Exome Aggregation Consortium) databases

Variant No	HGVS.c	1000 Genomes Project (MAF)		ExAC Population (MAF)	
		Global	African	Global	African
1	c.1268G > T	N/A	N/A	N/A	N/A
2	c.556dupC	N/A	N/A	N/A	N/A
3	c.563A > C	N/A	N/A	N/A	N/A

often masked by a high burden of infectious diseases, low diagnostic rates, lack of population knowledge of familial colorectal cancer and a low life expectancy. Given the competing priorities for resources, universal screening in Uganda may not be practical. Hence knowledge of these mutations which are responsible for CRC will be useful in the identification of high-risk individuals and this will be an important aspect of CRC prevention programmes in Uganda.

Conclusions

In this study, we contribute new clinicopathological information and genetic variation data to CRC patients in Uganda. In this paper, the genetic data provided represent a valuable resource for CRC in an underinvestigated indigenous population in East Africa. Future studies are required to improve the characterization of Ugandan CRC cases and improve evidence-based management of this disease.

Study limitations

Since we only used colorectal cancer tissues for analyses, we could not confirm whether the genetic mutations and/or variations were present in the germline or were purely somatic. In this part of the study, we did not perform immunohistochemical staining or functional studies to investigate further the effect of the described mutations on the expression levels of the corresponding proteins. To overcome the influence of antigen degradation of archival material, a high standard of laboratory testing was followed together with the maintenance of a short period of storage of specimens. Mutations of uncertain significance remain a challenge in many clinical studies. This is because they make up the majority of mutations but their exact penetrance remains unknown. For this study, we used a set of nine [9] in-silico tools to predict the consequences and although their results are not a gold standard, they provide valuable insight into the possible consequence of the candidate mutations which are recommended for further study.

The interpretation of novel mutations needs to be approached with caution, and the lack of functional studies or clinical correlation for these mutations is a significant limitation. We could not distinguish between cancer mutations and naturally occurring mutations in our population as we did not recruit controls in our study. With respect to the APC gene, the analysis was limited to only a small portion of this gene. The relation between smoking, alcohol consumption and family history and the presence or absence of a mutation were not determined in this study.

The small sample size may not capture all the possible mutations in colorectal cancer in our population however, our results provide a reasonable insight into some of the main genetic mutations involved in colorectal carcinogenesis in Ugandan patients. Since many patients in Uganda present at an advanced stage with CRC in hospital then a potential selection bias may have been introduced due to a smaller number of early-stage CRC observed in our study population.

Finally, underestimation or overestimation of the stage of CRC was another limitation. In developing low-income countries, especially in rural parts of the country, CT scanning is largely inaccessible. In the years 2008–2015 many patients had a CT abdomen and pelvis. With inadequate high-precision staging capacity, the stage at diagnosis was likely to be underassessed or over assessed. Since the stage was radiological and not pathological this also likely underassessed the CRC TNM stage. Proper CRC tumour staging depends on pathological examination of a minimum number of twelve lymph nodes in the colorectal specimen. Since there was a radiological and not a pathological assessment of assessment of lymph node involvement hence underestimating or overestimating the stage of the CRC.

Abbreviations

APC	Adenomatous polyposis coli
COSMIC	Catalogue of Somatic Mutations in Cancer
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
ExAC	Exome Aggregation Consortium
MAF	Minor allele frequency
SNP	Single nucleotide polymorphism
SD	Standard deviation
HGVS.c	Human genome variation society, coding
HGVS.p	Human genome variation society, protein

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Authors' contributions
RW conceived the concept, collected data, participated in the analysis and wrote the paper. RM extracted DNA from colorectal cancer tissue samples. CW designed and performed mutation analysis and DNA sequencing. FEK and ST carried out bioinformatics analysis of the variant data. JK carried out the statistical analysis. HW, MO and IT carried out critical reviews of the manuscript for intellectual content. All authors approved the final manuscript.

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Availability of data and materials
The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Higher Degrees Research and Ethics Committee, School of Biomedical Sciences, College of Health Sciences, Makerere University (Approval No: SBS-630) and Ugandan National Council for Science and Technology (Approval No: HS-2574). All participants who were recruited prospectively were informed of the purpose of the study, and they endorsed the informed consent forms prior to participation in this study. For the retrospective arm of the study, data were abstracted from the case files in the respective hospitals. Therefore, a waiver of consent was obtained from the Higher Degrees Research and Ethics Committee, School of Biomedical Sciences, College of Health Sciences, Makerere University, to access the data and perform the experiments on the tissue block samples. The patient data, which were accessed from the medical case files in the respective hospitals, were anonymized and maintained with confidentiality. The conduct of the study was in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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