ORIGINAL ARTICLE

Kristen Rat Sarcoma Viral Oncogene Mutations in Colorectal Carcinomas at the University Teaching Hospital in Lusaka, Zambia

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ABSTRACT

Background: The second leading cause of cancer-related deaths worldwide is colorectal cancer. With an incidence rate of 4.8 per 100,000, this is Zambia's sixth most prevalent cancer;

Methods: This laboratory-based, cross-sectional study examined the frequency of Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation and its association with prognostic factors in colorectal carcinoma cases from the University Teaching Hospital-Adult Hospital (UTHs), Lusaka, Zambia;

Results: Thirty (30) formalin-fixed paraffin-embedded (FFPE) samples collected between June 2017 and June 2018 were sent to the Lancet laboratories and analyzed for KRAS mutations (codons 12 and 13). One FFPE block did not meet the inclusion criteria and was excluded. The demographic and clinicopathological data were analyzed using STATA 12. Males outnumber females by 20 to nine. The average age of the patient was 45 ± 16 years. The rectum was the location of 44.8% of the tumors, with the majority being conventional adenocarcinoma (CAC) (65.6 %). All cases (100%) had advanced-stage (stages 3 and 4) disease; however, only 27.6% of patient tumors exhibited lymphovascular invasion. KRAS mutation was detected in 11 (37.9%) cases and mainly in left-sided tumors (62.5%). KRAS mutations were only detected in CAC and serrated adenocarcinoma subtypes. No significant associations were observed between the KRAS mutation status and tumor or patient's clinical and sociodemographic factors;

Conclusion: We advocate for incorporating KRAS mutation testing into the standard of care for treating colorectal cancer.

INTRODUCTION

Colorectal adenocarcinoma is the most prevalent digestive tract cancer. It accounts for approximately 9.4% of all cancer deaths worldwide.[1] The incidence of colorectal cancer is highest in industrialized nations like Canada and lowest in sub-Saharan Africa. In 2012, the age-adjusted incidence rate per 100,000 people in Canada, South Africa, and

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Zambia was 35.2%, 12.2%, and 4.5%, respectively.[2] There are four genetic instability mechanisms associated with colorectal cancer: chromosomal instability (CIN), microsatellite instability (MSI), the CpG island methylator phenotype (CIMP), and global DNA hypomethylation.[3–5] Eighty-five percent of colorectal cancers are associated with the CIN pathway, characterized by aneuploidy and loss of heterozygosity.[6] Adenomatous Polyposis Coli, p16, p53, and DCC tumour suppressor genes are inactivated during colorectal carcinogenesis, followed by the activation of mutations in the Kirsten rat sarcoma viral oncogene homolog (KRAS) gene. KRAS is a member of the small GTPase superfamily encoded by a gene from the RAS gene family in mammals.[7] The KRAS gene is located on chromosome 12's short arm (12p12.1)[8]. 30–40% of colorectal carcinomas contain a somatic activating mutation in the KRAS oncogene, most of which occurs in codons 12 and 13 of exon 2 (G > A transition in codon 12)[7,9]. KRAS mutations through the RAS/RAF/MEK/ERK signalling pathway promotes liver metastasis [10] in CRC patients. These mutations have also been seen to lead to overexpression of VEGF in about 50% of CRC patients.[11]

The treatment of colorectal cancer is contingent upon the KRAS gene mutation. Studies have shown that 30% to 50% of colorectal patients with a KRAS mutation are more likely to respond to anti-epidermal growth factor receptor (anti-EGFR) treatment, while up to 60% of patients with a wild-type KRAS gene do not respond to the same treatment.[12,13] Some studies, such as the CRYSTAL trial, OPUS phase II, and EVEREST study, have shown that anti–EGFR monoclonal antibodies, such as cetuximab, provide no benefit to colorectal cancer patients with a KRAS mutation.[12] Patients with KRAS mutations who received FOLFOX plus cetuximab performed worse in the OPUS study than those who received FOLFOX alone.[14] These outcomes were comparable to previous research on non-small-cell lung cancer.[15,16] TKIs have been used in CRC patients targeting the VEGF and PDGF receptors in KRAS mutated patients.[17] FOLFOX with cetuximab is administered to some colorectal cancer patients in Zambia, regardless of their KRAS status. This is mainly due to the high cost of testing for the presence of KRAS mutations in the samples of these CRC patients. The heterogeneity of colorectal adenocarcinoma necessitates an individualized treatment approach that is cost-effective.

This study aimed to determine the frequency of KRAS mutations in colorectal cancers found in indigenous Zambians and whether these mutations were associated with colorectal carcinoma's histopathology. The second objective was to identify any association between the diagnosis of a KRAS mutation and the patient's clinical or sociodemographic status and estimate the number of colorectal cancer patients who might benefit from anti-EGFR monoclonal therapy.

METHODS

This cross-sectional study collected thirty (30) formalin-fixed paraffin-embedded (FFPE) patient tumour specimens with a histological diagnosis of colorectal carcinoma diagnosed between June 2017 and June 2018. These specimens were identified using the Data Intensive Systems and Applications (DISA) laboratory information management system at UTHs in Lusaka, Zambia. The blocks of FFPE specimens were identified and retrieved. Excellence in Research and Ethics and Science (ERES) and the adult hospital at UTH permitted the study to be conducted (Ref No. 2019-Jan-010).

Due to the rarity of colorectal cancer, all available FFPE tissue samples were analysed for this study. The study included all tissue blocks from colon and rectal biopsies, resections, and colectomies with a histological diagnosis of carcinoma. Exclusion criteria included: i. tissue blocks with tumours comprising less than 30 % of total tissue; ii. Extensive autolysis or necrosis; and iii. Prior neoadjuvant chemotherapy or radiotherapy. One specimen had to be excluded due to this exclusion criterion. This left 29 cases to be investigated.

Five millimetres (5mm) thick tissue sections were stained with haematoxylin and eosin (H&E). A

registrar and a pathologist conducted the histopathological evaluation. The WHO histological classification was utilized to characterize the tumour's subtype, grade, and location [18]. In addition, peritumoral lymphocytes and lymphovascular invasion were evaluated. The final diagnosis was arrived at by consensus by two pathologists.

Mucinous carcinoma is a tumour in which greater than fifty percent (50%) of the lesion consists of extracellular mucin pools containing blatantly malignant epithelial cells. Signet ring adenocarcinoma is a type of adenocarcinoma characterized by the presence of intracytoplasmic mucin in at least half of the tumour cells as well as the displacement and reshaping of the nucleus. Serrated carcinoma was defined as a tumour with at least six of the following seven features: ii. Eosinophilic or clear cytoplasm; iii. An abundance of cytoplasm; iv. Vesicular nuclei with peripheral chromatin condensation and a single prominent nucleolus; v. Clear nucleoli; vi. An absence of necrosis (or 10 % necrosis); and vii. Intracellular or extracellular mucin. [18]defined a conventional adenocarcinoma(CAC) as a malignant epithelial neoplasm that could not be classified into a particular histologic subtype.

If the tumour was discovered in the caecum, ascending, or transverse colon, it was classified as proximal or right-sided. If colon cancer was between the splenic flexure and the sigmoid colon, it was on the left side, and if it originated in the rectum, it was classified as a rectaltumour[18].

There are two grades for colorectal carcinoma: low grade and high grade. Formally, low-grade tumours were moderate to well-differentiated, whereas high-grade tumours were poorly differentiated.[18].

Lymphovascular tumour invasion was defined as the presence of single tumour cells or clusters of tumour cells within lymphatic channels. Immunohistochemistry to highlight lymphatic channels was unavailable. [18] The tumour was staged using clinical information from the patient's medical records and radiological reports.

The tissue blocks containing over 30% tumour were sent to the Lancet laboratories for KRAS mutation analysis. The KRAS mutation test detected seven somatic mutations in codon 12 and codon 13 of the KRAS gene using the Cobas 4800 system. COSMIC IDs 516, 517, 518, 520, 521, 522, and 532 were assigned to the identified seven mutations[19].

Data were collected, cleaned, and categorized using Excel, SPSS 25, and GraphPad Prism 8 was used for analysis. The respective percentages of tumours and variables are displayed in tables 1 and 2. Distribution and outliers were analysed for all continuous variables. Age attained according to a normal distribution ($W = 0.972$; $p = .648$; Shapiro-Wilk). Therefore, a student T-test was used to determine age difference means between the wild-type and mutant KRAS genotype groups. A chi-square association analysis was conducted between demographic, histologic, and KRAS genotype parameters. Fisher's exact test was reported when the chi-square assumptions were not met. The reported significance level was $P > .05$.

RESULTS

Using convenience sampling, we retrieved thirty cases diagnosed within the sampling period. One case could not be analysed due to insufficient tissue. Table 1 summarizes the retrieval and analysis of 29 tumours diagnosed as colorectal adenocarcinoma. There were 20 men (69.0 %) and nine women (31.0%), with a mean age of 45.2 ± 15.9 years.

A review of medical records revealed that the rectum was the site of mosttumours (44.8 %). On the left and right sides of the colon, tumours were reported at 20 % and 17.2 %, respectively. Histopathology subclassified the colorectal cancers as CAC in 19 cases (65.6%), serrated adenocarcinoma (SAC) in five patients (17.2%), mucinous adenocarcinoma (MAC) in three patients (10.4%), and signet ring adenocarcinoma (SRS) in two patients (6.9%). When the files of 25 patients were examined, it was discovered that in 25 instances, both clinical evaluation and radiological staging of the tumours had been performed. At the time of assessment, all

Table 1: Demographic and histologic statistics

All cases (N=29)	Overall
Age*	
Mean ± SD [Range]	45.2 ± 15.9 [13-75]
Sex	
Male	20 (69.0)
Female	9 (31.0)
Tumour location	
Caecum	0 (0.0)
Ascending colon	1 (3.5)
Transverse colon	4 (13.8)
Descending colon	0 (0.0)
Sigmoid colon	6 (20.7)
Rectum	13 (44.8)
Unknown	5 (17.2)
Right	5 (17.2)
Left	6 (20.7)
Rectum	13 (44.8)
Unknown	5 (17.2)
Histologic subtype	
Conventional adenocarcinoma (CAC)	19 (65.5)
Serrated adenocarcinoma (SAC)	5 (17.2)
Mucinous adenocarcinoma (MAC)	3 (10.4)
Signet ring adenocarcinoma (SRS)	2 (6.9)
Tumor grading	
I	6 (20.7)
II	8 (27.6)
III	15 (51.7)
Lymphovascular invasion	
Absent	21 (72.4)
Present	8 (27.6)
Tumor-infiltrating lymphocytes	
Absent	20 (69.0)
Present	9 (31.0)
Tumor stage	
III	17 (58.6)
IV	8 (27.6)
Unknown	4 (13.8)

* n=28

patients had advanced (stage III or IV) disease. The staging revealed that 17 (58.6%) and 8 (27.6%) patients had clinical stages III and IV, respectively. Based on histology, the tumour was classified as poorly differentiated (grade 3) in 51.7% of cases, moderately differentiated (grade 2) in 27.6% of cases, and well-differentiated (grade 1) in the remaining (20.7%) cases. There were only eight patients (27.6%) with lymphovascular invasion. Slides stained with Haematoxylin and Eosin (H&E) revealed peritumoral infiltrating lymphocytes in nine (31%) of the 29 patient samples. The second table presents the frequency of tumour subtypes by sex, age, tumour location, and histopathologic characteristics. In the rectum, we observed all tumoursubtypes (CAC, SAC, MAC, and SRS). Two cases of the SRS subtype were detected in the rectal region. Only tumours in the rectosigmoid area constituted MAC in three patients (10.3%).

Cases (N=29)	CAC *N = 19*	SAC *N = 5*	MAC *N = 3*	SRS *N = 2*
Age				
Mean ± SD	45.1 ± 15.1 [22-71]	46.2 ± 4.1 [42-51]	63.0 ± 13.1 [49-75]	17.0 ± 5.7 [13-21]
Sex				
Male	13 (68.4)	3 (60.0)	2 (66.7)	2 (100.0)
Female	6 (31.6)	2 (40.0)	1 (33.3)	0 (0.0)
Tumor location				
Caecum	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ascending colon	1 (5.3)	0 (0.0)	0 (0.0)	0 (0.0)
Transverse colon	4 (21.0)	0 (0.0)	0 (0.0)	0 (0.0)
Descending colon	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Sigmoid colon	4 (21.0)	1 (20.0)	1 (33.3)	0 (0.0)
Rectum	6 (31.6)	3 (60.0)	2 (66.7)	2 (100.0)
Unknown	4 (21.0)	1 (20.0)	0 (0.0)	0 (0.0)
Right	5 (26.3)	0 (0.0)	0 (0.0)	0 (0.0)
Left	4 (21.0)	1 (20.0)	1 (33.3)	0 (0.0)
Rectum	6 (31.6)	3 (60.0)	2 (66.7)	2 (100.0)
Unknown	4 (21.0)	1 (20.0)	0 (0.0)	0 (0.0)
Tumor grading				
I	3 (15.8)	3 (60.0)	0 (0.0)	0 (0.0)
II	5 (26.3)	2 (40.0)	1 (33.3)	0 (0.0)
III	11 (57.9)	0 (0.0)	2 (66.7)	2 (100.0)
Lymphovascular invasion				
Present	4 (21.0)	1 (20.0)	2 (66.7)	1 (50.0)
Tumor-infiltrating lymphocytes				
Present	5 (26.3)	2 (40.0)	2 (66.7)	0 (0.0)
Tumor stage				
III	12 (63.2)	2 (40.0)	2 (66.7)	1 (50.0)
IV	4 (21.0)	2 (40.0)	1 (33.3)	1 (50.0)
Unknown	3 (15.8)	1 (20.0)	0 (0.0)	0 (0.0)

MAC: Mucinous adenocarcinoma; SAC: Serrated adenocarcinoma; SRC: Signet ring adenocarcinoma; CAC: Conventional adenocarcinoma.

Each case was analysed for mutations in the KRAS gene. Table 3 displays the association between KRAS mutation and patient and tumour characteristics. KRAS mutations were identified in 11 (37.0%) of these cases. Sixty percent (60.0%) of right-sided colorectal tumours contained KRAS mutations, followed by rectum-based tumours (40 %). We did not detect any KRAS mutations in six cases of left-sided colon cancer. Only CAC (47.4 %) and SRS (40 %) colorectal cancer subtypes contained KRAS mutations. KRAS mutations were identified in all tumour grades, irrespective of the presence or absence of peritumoral lymphocyte infiltration. In stages III and IV tumours, the KRAS mutation was observed 29.4 % and 50.0 % of the cases, respectively. There was no association

All participants (N=29)	**Wild type (n=18)** n (%)	**Mutant (n=11)** n (%)	**Association** Statistics (p-value)
Age of Participants			
Age: Mean ± SD	43.3 ± 17.4	48.7 ± 12.9	1.184 (26) (.398)[1]
Sex			
Male	13 (65.0)	7 (35.0)	0.235 (.628)
Female	5 (55.6)	4 (44.4)	
Tumor location			
Caecum	0 (0.0)	0 (0.0)	
Ascending colon	0 (0.0)	1 (100.0)	
Transverse colon	2 (50.0)	2 (50.0)	
Descending colon	0 (0.0)	0 (0.0)	N/a
Sigmoid colon	6 (100.0)	0 (0.0)	
Rectum	8 (61.5)	5 (38.5)	
Unknown	2 (40.0)	3 (60.0)	
Transverse vs. Rectum			> .999*
Right	2 (40.0)	3 (60.0)	R vs. L: N/a
Left	6 (100.0)	0 (0.0)	R vs. Re: .329*
Rectum	8 (61.5)	5 (38.5)	L vs. Re: N/a
Histologic type			
Conventional adenocarcinoma (CAC)	10 (52.6)	9 (47.4)	
Serrated adenocarcinoma (SAC)	3 (60.0)	2 (40.0)	N/a
Mucinous adenocarcinoma (MAC)	3 (100.0)	0 (0.0)	
Signet ring adenocarcinoma (SRC)	2 (100.0)	0 (0.0)	
SAC vs. CAC			>.999*
Tumor grading			
I	4 (66.7)	2 (33.3)	I vs. II: .592*
II	3 (37.5)	5 (62.5)	I vs. III: > .999*
III	11 (73.3)	4 (26.7)	II vs. III: .179*
Lymphovascular invasion			
Absent	14 (66.7)	7 (33.3)	.394*
Present	4 (50.0)	4 (50.0)	
Tumor-infiltrating lymphocytes			
Absent	10 (50.0)	10 (50.0)	.096*
Present	8 (88.9)	1 (11.1)	
Tumor stage			
III	12 (70.6)	5 (29.4)	
IV	4 (50.0)	4 (50.0)	.394*
Unknown	2 (50.0)	2 (50.0)	

[1]: T-test report [f (df), p-value]; *: Fisher's exact test.

between KRAS mutation and the patients' age (1.184 (26), p = .398) or sex ($?^2$ (2) = 0.235, p = .628). There was no association between the location of colorectal adenocarcinomas in the transverse colon as opposed to the rectum and the presence of a KRAS mutation (fishers exact, p > .999). Even though KRAS mutations were most prevalent in CAC and SRS colorectal carcinoma subtypes, there was no association between KRAS mutations and carcinoma subtypes (fishers exact, p > .999). There was no association between KRAS mutations and tumour grade (I versus II: Fisher exact, p = .592; I versus III: P > 0.999; II versus III: P =.179). There was no association between the KRAS mutation and the presence of lymphocytes infiltrating the tumour (p = .096), lymphovascular invasion (p = .394), or tumour stage (p = .394).

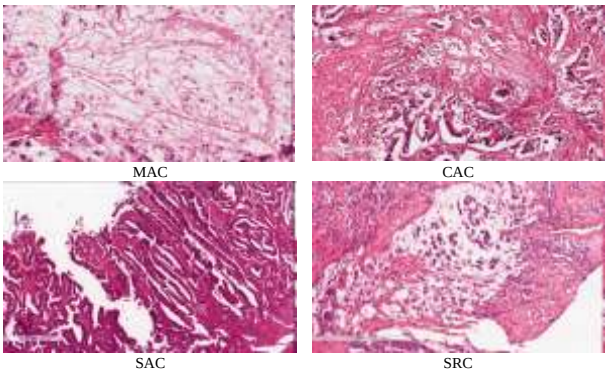

Figure : Variants of colorectal carcinoma observed in our study. Mucinous adenocarcinoma (MAC), Conventional adenocarcinoma (CAC), Serrated adenocarcinoma (SAC), and signet ring adenocarcinoma (SRC).

DISCUSSION

The objective of this study was to determine the frequency of KRAS mutations in colorectal cancers among Zambian natives and whether these mutations are associated with the histopathology of colorectal carcinoma or patient factors. KRAS mutations were more common in advanced-stage tumours and on the right side of the colon, where advanced-stage tumours were more prevalent. In most cases, colorectal carcinomas were found in the rectum and at an advanced clinical stage. There was no association between KRAS mutations and tumours' histology, clinical, or sociodemographic features.

In line with previous research indicating an increasing trend of colorectal cancer diagnoses among middle-aged men in developing countries[20,21], we report that roughly two-thirds of males diagnosed with colorectal carcinoma in our study population in Zambia were in the young and middle age groups (below 50 years of age). A similar pattern was observed in a retrospective study conducted between 2007 and 2015 at the Cancer Diseases Hospital in Lusaka, Zambia, which reported a mean age at diagnosis of 48.6 years and a preponderance of males (62.0%). South African research indicates that colorectal cancer is more prevalent among young, male, and black populations.[22] In a 2011 study by Liu et al., the average age of patients with colorectal carcinoma was 59.8 years, and 49.0 % were male.[23] We observed a similar male predominance pattern among the subtypes of colorectal cancer. According to our knowledge, we are the first to classify colorectal cancer subtypes by sex and age. We report that males closer to 50 years of age were diagnosed with CAC, SAC, and MAC, whereas two males between 13 and 21 years of age presented with SRS. There was no statistical difference between the wild-type and mutant groups regarding age and sex.

It is crucial to classify the location of colorectal cancer, as tumours on the left and right sides of the colon differ in genetic composition, overall survival, and disease progression [24]. It has been widely reported that mutations in the chromosomal instability pathway, such as KRAS, cause left-sided tumours.[24] Findings in our study of tumours on the left side of the colon (20.7%) and the rectum (44.8 %) are like previously described results from Zambia that showed colorectal cancer patients between 2007 and 2015 had tumours located primarily in the rectum (53 %) and sigmoid colon (13.4%)[25]. Similarly, a Tanzanian study [26] found that 54.8 % of tumours in patients originated in the rectosigmoid region. In this study, the most (65.6%) common histopathological subtype of colorectal carcinoma was CAC.

Similarly, CACs were reported to be the most frequent subtypes in China and the United States at

93.7% and 88.8%, respectively.[27,28]Most cases had advanced clinical stages (51.7%) and poor differentiation (51.7%)[29] also reported poorly differentiated CAC lesions in Ugandans younger than 50 years of age, while another study conducted in Tanzania reported somewhat similar findings, with the majority of cases being advanced stage (II-IV) adenocarcinoma (98%), and at a higher prevalence rate in their study population [26]. The lack of early colorectal cancer screening services in our setting may have contributed to the presence of advanced disease (stages III and IV) in the evaluated patients.

About 10-15% of colorectal carcinomas are classified as MAC.[30,31] In contrast to what was reported in Uganda, where the majority of MAC cases were diagnosed in the younger population (50 years or younger), the majority of MAC cases in our study were male and older than 50 years of age (63 ± 13.1 years).[29] The serrated pathway (SAC) accounts for approximately 7.5% of all colorectal cancers, making it the second most prevalent colorectal cancer pathway.[32] This study's second most prevalent colorectal cancer was SAC, accounting for 5 (17.2%) cases. Four of these cases occurred in the rectosigmoid area, while one case had an unknown location. Three (60%) of the five cases were reported in males. This was in stark contrast to the findings of Stefanius *et al.*[33], in which SAC was more frequently observed in females (59.5%), and the tumours were more often located on the proximal colon (57.1%) as opposed to the distal colon (14.3%) and rectum (28.9 %). These differences could be attributed to the small sample size in our study.

Signet ring cell carcinoma (SRC) is an uncommon subtype of colorectal cancer with a poor prognosis and no clear putative pathogenesis.[34] Consistent with previous research, this study diagnosed SRS in 6.9% of cases. Previous research conducted in the United States between 1998 and 2002 found that SRS was predominantly diagnosed in a younger population, 17.0 5.7 years old.[28]Our findings are similar to reports by Kakar *et al*[35], which found that SRC was most prevalent in males, 72.7% of cases in our study were male, and all instances of SRC were located in the rectum.

Lymphovascular invasion in colorectal cancer is crucial in determining metastasis development.[36,37] We anticipated a small proportion of lymphovascular invasion in our study because none of the cases reported metastasis. We only observed lymphovascular space invasion in a third of the cases, which is accurate. Thirty percent (30%) of patients in South Africa also showed evidence of lymphovascular invasion.[38] Nonetheless, the absence of confirmation studies employing immuno-histochemistry techniques to highlight lymphovascular bundles is a significant limitation.

This is the first study to report the status of KRAS mutations in Zambian cases. According to Arrington *et al*[39] and Margetis *et al*[40]KRAS mutations are a critical genetic alteration associated with the progression of adenoma to colorectal cancer. Patients with wild-type KRAS have higher overall response rates and more prolonged progression-free survival than patients with the mutation.[41–43] In our study, the overall frequency of KRAS mutations was 37.9 %, compared to 44.4 % and 48.4 %, respectively, in the Arabian Peninsula and the United States[44] and 31.3 % in Egypt.[45] The association between KRAS mutation and colorectal adenocarcinoma subtypes and sociodemographic factors is presented in Table 3. Our research found no significant association between the patient's age, sex, tumour location, and histopathology tumour subtype.The frequency of KRAS mutations in SAC was determined to be 40% by our research. Comparable percentages for African-Americans, Americans, and Moroccans are 37 %, 45 %, and 36.7 %, respectively.[33,46,47] It was predicted that SAC tumours would have a lower frequency of KRAS mutations than CAC tumours. Previous research shows that SAC tumours contain BRAF mutations more frequently than KRAS mutations.[33] In our study, none of the three cases of MAChad a KRAS mutation, in contrast to the 57.1 % and 65 % reported in the United States.[27,48] These variations may be attributable to racial, genetic, and environmental differences between the studied populations.

The small sample size and limited data captured by patient records aresome of the study's limitations; as

a result, a recommendation is made to improve patient data entry in medical data management systems. In addition, it is necessary to analyse samples gathered over a more extendedtime. Since endoscopic biopsies were used to obtain most of the samples, extensive histopathological characteristics could not be ascertained on all patient biopsy slides.

CONCLUSION

About one-third of patients had KRAS mutations. Mutations were more prevalent in advanced-stage tumours and on the right side of the colon, where advanced-stage tumours were more prevalent. Most colorectal carcinomas in our cases were located in the rectum and had an advanced disease stage. There was no association between KRAS mutations and tumour histology or clinical and sociodemographic information. We require a large prospective study on KRAS mutations in colorectal carcinomas in Zambia to inform disease treatment.

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Author Contribution

EPS, MM, SNS, and PJ, conceived and analysed all study data. EPS and PJ conducted the histology examination. EPS and SNS wrote the first draft of the manuscript, while PJ oversaw all aspects of the study. All authors reviewed and approved the manuscript.

Competing Interests

All authors declare no conflict of interest.

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