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Distinct molecular features of colorectal cancer in Ghana

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Abstract

Objectives—While colorectal cancer (CRC) is common, its incidence significantly varies around the globe. The incidence of CRC in West Africa is relatively low, but it has a distinctive clinical pattern and its molecular characteristics have not been studied. This study is one of the first attempts to analyze molecular, genetic, and pathological characteristics of colorectal cancer in Ghana.

Methods—DNA was extracted from microdissected tumor and adjacent normal tissue of 90 paraffin blocks of CRC cases (1997–2007) collected at the University of Ghana. Microsatellite instability (MSI) was determined using fragment analysis of ten microsatellite markers. We analyzed expression of mismatch repair (MMR) proteins by immunohistochemistry and sequenced exons 2 and 3 of *KRAS* and exon 15 of *BRAF*.

Results—MSI analysis showed 41% (29/70) MSI-High, 20% (14/70) MSI-Low, and 39% (27/70) microsatellite-stable (MSS) tumors. Sequencing of *KRAS* exons 2 and 3 identified activating mutations in 32% (24/75) of tumors, and sequencing of *BRAF* exon 15, the location of the common activating mutation (V600), did not show mutations at codons 599 and 600 in 88 tumors.

Conclusions—Our study found a high frequency of MSI-High colorectal tumors (41%) in Ghana. While the frequency of *KRAS* mutations is comparable with other populations, absence of *BRAF* mutations is intriguing and would require further analysis of the molecular epidemiology of CRC in West Africa.

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Conflict of interest statement

The authors do not have any conflicts of interest.

Keywords

Colorectal cancer; MSI; *BRAF*; Ghana; Microsatellite instability; West Africa

1. Introduction

Colorectal cancer is the fourth-most common cancer in men and the third-most common cancer in women worldwide, with significant variation between different parts of the globe [1]. Over the past few decades, incidence rates of colorectal cancer have increased around the world, probably due to adoption of western lifestyle and behaviors by developing countries [2]. The hypothesis of westernization could explain this rise in Eastern European countries, Asia, and some countries of South America. The incidence of colorectal cancer in Africa, estimated from registries in South Africa, is lower than in Europe, North America, and Oceania. Unfortunately, little is known about the epidemiology and biology of colorectal cancer in Native Africans from West Africa. Recent publications from Nigeria and Ghana show that colorectal cancer in West Africa has a distinctive pattern with young age of onset and predominantly left-sided tumors [3,4].

Colorectal cancer is developed through different pathways including chromosomal instability, microsatellite instability (MSI) due to inherited or acquired defects in DNA mismatch-repair (MMR) genes, and the CpG island methylator phenotype (CIMP) [5]. There are also rare hereditary colorectal cancer syndromes caused by mutations *APC*, *TP53*, *STK11*, *PTEN* and other genes. Colorectal tumors with instability at microsatellite sites (MSI-High) have deleterious mutations in MMR genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) or CIMP. Chromosomal instability (CIN) phenotype caused by genetic defects in p53, *KRAS*, *APC*, *EGFR*, *BRAF* and other genes [6]. Recent analysis also showed frequent mutations in *SMAD4*, *PIK3CA*, *ARID1A*, *SOX9* and *FAM123B*, recurrent copy-number alterations of *ERBB2* and *IGF2*, as well as fusion of *NAV2* and *TCF7L1* [7]. Despite the progress in research of genetic and molecular factors involved in the development of colorectal cancer, our knowledge about somatic mutations in oncogenes and tumor-suppressor genes is far from complete. This is particularly true for colorectal cancer in patients of African descent, where molecular and genetic factors are understudied and may be different from the molecular landscape of colorectal cancer in white populations.

The spectrum of molecular changes in colorectal tumors has not been characterized in black West African populations. Data on the molecular pathology of colorectal cancer in African Americans are also scarce [8-12]. Previously published studies showed 15–45% frequency of MSI-High tumors in African-American patients [8-12], compared to MSI-High colorectal tumors in 10% of white [13] and 15% of mixed populations [14]. MSI-High tumors in African Americans were found more frequently in women with a mean age of 66–69 years at diagnosis in the proximal colon (right side) [8-12]. *BRAF*-activating mutations have been found in high proportions of MSI-High colorectal tumors in African Americans (23–39%) but not in microsatellite-stable (MSS) tumors (0–4%) [8,11,12]. *KRAS* mutations were detected slightly more frequently in MSS tumors (34–36%) than in MSI-High tumors (21–24%) [8,12]. It has been shown that African-American colorectal cancer patients with MSS

tumors have a higher proportion of *KRAS* mutations than white patients [8]. It also has been demonstrated that African-American colorectal cancer patients have lower survival rates [15,16]. Different genetic risk variants are associated with colorectal cancer in African Americans than white patients [17].

The aim of this study was to investigate molecular characteristics of colorectal cancer in Ghana and compare results to the published data on molecular biology of colorectal cancer in patients of African ancestry.

2. Methods

2.1. Selection of samples and processing

All consecutive colorectal cancer cases with available formalin-fixed paraffin-embedded (FFPE) pathology blocks collected at the University of Ghana Medical School (Ghana, Accra) over an 11-year period (1997–2007) were included in the study. These samples were also part of a published study of tendencies in colorectal cancer morbidity in Ghana [3]. Ninety FFPE blocks of colorectal tumors were retrieved and sent to the University of Michigan for analysis. Ten 5 µm recuts and H&E slides were prepared from each block. H&E slides were used by a pathologist (J.K.G.) at the University of Michigan to perform full pathology review and to determine areas with >70% tumor cellularity and areas of adjacent normal tissue. Five recut slides were microdissected, and DNA was extracted from the tumor and from normal tissue using MoBio BiOstic FFPE Tissue DNA Isolation Kit (MoBio, Carlsbad, CA). Only 71 samples had both tumor and normal tissue. Seventeen samples had tumor only and were not eligible for MSI testing, although they were used for *KRAS* and *BRAF* sequencing.

2.2. MSI testing

The protocol for MSI testing was developed in our laboratory and published elsewhere [18]. Microsatellite marker loci were amplified using three multiplex PCR mixes as follows: MIX 1 (D10S197, BAT26, beta-catenin); MIX 2 (D18S58, BAT40, D2S123); MIX 3 (D17S250, BAT25, TGF-b-RIIF, and D5S346F). All three PCRs were performed using the same conditions: 10 min of initiation step at 95 °C followed by three cycles at 95 °C for 1 min, 57 °C for 1 min, 72 °C for 1.5 min, and then 40 cycles at 95 °C for 1 min, 51 °C for 1 min, 72 °C for 1.5 min, and finished by the final elongation step at 72 °C for 10 min. PCR fragments were detected by capillary electrophoresis on ABI370 (Applied Biosystems, Foster City, CA). Chromatograms were analyzed using GeneMarker v1.95 software (SoftGenetics, LLC, State College, PA). Patterns of microsatellite markers in tumor and adjacent normal tissue were compared to find changes in the length of the markers. Only tumors with at least one mononucleotide marker and a minimum of three available markers were considered for analysis. Tumors with 30% or more of unstable markers were called MSI-High, less than 30% – MSI-Low, and tumors with all stable markers were called microsatellite stable (MSS). To verify the results of our MSI testing method we repeated MSI analysis using MSI Analysis System, Version 1.2 (Promega, Fitchburg, WI).

2.3. BRAF and KRAS sequencing

BRAF exon 15 and KRAS exons 2 and 3 were amplified using following PCR primers: BRAF-F_5'-TTCATGAAGACCTCACAGTAAAAA-3'; BRAF-R 5'-TCCACAAAATGGATCCAGAC-3'; KRAS2-F_5'-AGGCCTGCTGAAAATGACTG-3'; KRAS2-R_5'-GCTGTATCGTCAAGGCACTCT-3'; KRAS3-F_5'-TGGAGAAACCTGTCTCTTGA-3'; KRAS3-R_5'-AAAGAAAGCCCTCCCCAGT-3'. PCR conditions were 5 min at 95 °C; 40 cycles of 1 min at 95 °C, 1 min at 58 °C, and 1 min at 72 °C; and 10 min at 72 °C. PCR fragments were sequenced using ABI 3700 sequencer in both forward and reverse directions twice with positive and negative controls. Two independent researchers using Mutation Surveyor 2.61 and Sequencher 4.6 reviewed chromatograms.

2.4. Immunohistochemistry

Immunohistochemical staining was performed on the Leica BOND III system (automated machine) (Leica Microsystem, Buffalo Grove, IL) using the standard protocol with H1 (20) for MLH1 and MSH6; H2 (20) for MSH2 and PMS2. 5 µm recuts were deparaffinized, labeled with antibodies from Leica/Novocastro, RTU and counterstained with hematoxylin. The intensity of staining was interpreted as normal and loss of expression.

2.5. Statistical analysis

All statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC). Univariate analysis of continuous variables was performed using *t*-test, and categorical variables were analyzed using chi-square and Mantel-Haenszel tests. Logistic regression was used for multivariate analysis. In all analyses, *p*-values < 0.05 were considered statistically significant.

3. Results

3.1. Sample description

Of 90 samples retrieved for this study age and sex information was available for 89 patients (Table 1). Median age at diagnosis of colorectal cancer was 54 years with a range between 14 and 90 years. Of 89 subjects, 34% (30/89) were diagnosed with colorectal cancer before age 50, and 7% (6/89) were diagnosed at age 50. Number of female patients was slightly higher in the sample (*n* = 52) than male patients (*n* = 37). Tumor anatomical location data were available for 89 patients. Nearly 27% (24/89) of all tumors were located in the proximal colon (cecum, ascending colon, hepatic flexure, transverse colon), 23% (20/89) in the distal colon (descending colon, sigmoid), and 50% (45/89) of tumors were in the rectum. Only Astler–Coller staging was available: five patients had stage A and 11 patients stage B1 cancer (corresponding to AJCC Stage I), 24 patients had B2 (AJCC Stage II), 22 patients had C1 and 12 patients had C2 (AJCC Stage III), and 15 patients had stage D (AJCC Stage IV).

3.2. Pathology of colorectal tumors

All tumors included into the study were adenocarcinomas. Pathological data were not available for all tumors. Of 81 tumors, 25% (20/81) were well differentiated, 50% (41/81) were moderately differentiated, and 25% (20/81) were poorly differentiated. Tumor differentiation was not determined for 10% of the sample (9/90). Mucinous carcinomas were found in 14% (10/70), signet ring cell carcinomas were found in 3% (2/72) of cases and tumor necrosis in 57% (39/68) of samples with available data. Crohn-like inflammatory response, defined by a minimum of three lymphoid aggregates per section, was found in 57% (24/42) of cases. The count of tumor-infiltrating lymphocytes (TILs) in randomly selected high-power fields was available for 72 tumors with a mean value of 4.3 and range between 0 and 100. There was no statistically significant difference in the number of TILs between stable and unstable tumors (p -value = 0.58). Approximately 72% (31/43) of tumors had extensive pattern of growth, 21% (9/43) had invasive growth, and 7% (3/43) had both extensive and invasive growth. No pathological features described above were found to be significantly associated with MSI status.

3.3. MSI analysis

Of the 71 available pairs of colorectal tumor tissue and adjacent normal tissue, MSI testing worked on 70 pairs. We found 29 (41%) MSI-High tumors, 14 (20%) MSI-Low tumors, and 27 (39%) MSS tumors (Fig. 1 and Table 2). To validate the results of our MSI testing method, we repeated analysis on 24 randomly selected samples using Promega MSI Analysis System, Version 1.2. The concordance between MSI-H and MSS/MSI-L results from our method and Promega kit was 100%. Loss of heterozygosity (LOH) was detected for all markers (except *TGF-beta*), although at various frequencies. Marker D5S346 showed the highest LOH frequency: 31%; followed by D18S58 with 23% LOH. Other dinucleotide markers showed LOH in 14-16% of cases.

3.4. Analysis of BRAF and KRAS

We sequenced exons 2 and 3 of *KRAS* in 76 tumors and identified 23 (30%) activating mutations (G12D, G12V, G12C, G12S, G13D, Q61R, and Q61K). Surprisingly, sequencing exon 15 of *BRAF*, the location of the common activating mutation V600E, did not show any mutations at codons 599 and 600 in 88 tumor samples (Fig. 2). Sequencing was repeated twice from both directions and analyzed by two independent readers using Sequencher from GeneCodes (Ann Arbor, MI) and Mutation Surveyor from Soft-Genetics (State College, PA) to avoid misinterpretation.

3.5. Immunohistochemical analysis of MMR proteins

Immunohistochemical analysis of MMR proteins in colorectal tumors from Ghana was challenging; staining worked for only a limited number of samples. We believe that during preparation of paraffin blocks, formalin fixation was performed for an extended time period, which lead to excessive dehydration of the tissue. A sufficient number of recut slides was available for 66 tumors. MLH1 was adequately stained in 42 cases and showed loss of expression in 4 cases. MSH2 and MSH6 were stained in 48 and 51 tumors respectively and did not show loss of expression in any of the cases. We found loss of PMS2 expression in 4

cases out of 44 adequately stained tumors. Four out of five tumors with loss of MMR expression were MSI-High and one was MSS. Three tumors had loss of expression of both MLH1 and PMS2 (Fig. 3). One tumor had loss of MLH1 expression only, and one tumor had loss of PMS2 expression only.

4. Discussion

This study is one of the first attempts to analyze the molecular characteristics of colorectal cancer in West Africa. Rapidly growing population, life expectancy, and the number of treated colorectal cancer patients in Ghana can make this type of cancer a challenging healthcare problem in the future. Successful preparedness requires better understanding of tumor biology in Ghanaian populations and identification of molecular and genetic biomarkers of colorectal cancer in this population.

In addition to different clinicopathological features of Ghanaian colorectal tumors in comparison to white populations, we also demonstrate differences in molecular characteristics of the tumors, including higher incidence of MSI-High tumors and absence of *BRAF* mutations. Two studies reported very low to zero frequency of *BRAF* exon 15 mutations among Iranians [19,20]. It is noteworthy that the Iranian study population also had a higher frequency of distal tumors – similar to our study. The association of wild-type *BRAF* with distal tumor location is consistent with previous reports [21]. A recent meta-analysis of *BRAF*-activating colorectal cancer mutations in white populations showed a high prevalence (5%) of these mutations in MSS tumors and much higher prevalence (63.5%) in tumors with *MLH1* promoter hypermethylation, but low prevalence (1.4%) in tumors with mutations in MMR genes [22]. It is possible that MSI-High tumors without *BRAF* mutations in our study are Lynch syndrome cases, although the majority of cases in our study are left-sided, unlike right-sided Lynch syndrome observed in other populations. Attained expression of four MMR proteins in some of MSI-High tumors in our study raises a question about frequency of mutations in other proteins, such as PMS1 and TGFBR2, in Native Africans. A very small study of five randomly selected colorectal cancer patients from Nigeria identified two MSI-High tumors (40%) [23]. These results are very similar to the frequency demonstrated by our data (41%), although further studies with larger sample sizes are warranted. While MSI-High tumors in white patients are associated with proximal location of the tumor, younger age, and female gender, we did not find these associations in patients from Ghana. A relatively high number of left-sided carcinomas and the young age of one-third of the patients in our study raise questions about the prevalence and nature of Lynch syndrome and other colorectal cancer syndromes in Ghana.

Distribution of anatomical locations of colorectal tumors in our study is roughly similar to the distribution described in Nigeria [4], showing high frequency of rectal cancer (64–69%). It is difficult to explain the high prevalence of rectal adenocarcinomas in our sample, but similar trends have been noted in other studies from Ghana and Nigeria [4]. Possible reasons include higher detection rate of rectal cancer, influence of environmental factors, or genetic predisposition. In Asians mucinous colorectal cancer has been reported in 6–25% of cases, and it is associated with poor prognosis, young age of onset, and MSI-High tumors [24]. The relatively high frequency of mucinous tumors (27%) found in our study may partially

explain the high prevalence of MSI-High tumors in our results. We found signet ring carcinoma in 27% (3/11) of the patients younger than 30 years. These results are comparable with the high frequency (40–50%) of signet ring carcinomas in patients younger than 30 years in Nigeria [25].

It is noteworthy that MSI analysis of African populations is more challenging than in white populations where mononucleotide markers are less diverse and even can be pseudomonomorphic, as in the case of BAT26. We found more heterozygotes and fewer repeats in mononucleotide markers than observed in white populations, therefore MSI analysis in patients of African descent should be performed with caution. Our study has limitations related to the quality of pathological specimens and small sample size. As well, we were unable to test MLH1 promoter hypermethylation. These concerns should be addressed in future prospective studies of colorectal cancer in Ghana with well-organized preparation of pathological samples, assessment of germline predisposition, larger sample size, and *MLH1* methylation testing. We believe that investigation of the differences in tumor biology between black and white populations can advance the understanding of carcinogenesis and improve healthcare.

Our study analyzed molecular, genetic, and pathological characteristics of colorectal cancer in West Africa. The results showed important tumor biology peculiarities in colorectal cancer patients from Ghana; this knowledge may have important clinical value for all West African patients. Furthermore, characterization of the molecular landscape of colorectal tumors in different populations around the world may help to understand colorectal cancer pathogenesis.

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Fig. 1. Representative images of MSI analysis in Ghana colorectal tumors. Differences in fragment size and pattern between normal sample (upper part) and tumor (lower part) in BAT25 mononucleotide marker (A) and BAT26 mononucleotide marker (B).

Fig. 2. Representative chromatogram showing clear absence of BRAF V600 mutations in Ghana colorectal tumors as compared to positive control.

Fig. 3. Representative images of IHC staining of four MMR proteins in one of the tumors from Ghana. The staining of MSH2 (A) and MSH6 (C) are retained, but there is loss of staining of MLH1 (B) and PMS2 (D) in the same tumor.

Table 1

Demographic and clinicopathological characteristics of Ghana colorectal cancer samples by MSI status.

	All samples	MSS/MSI-Low	MSI-High	<i>p</i> -Value ^a
Overall	90(100%)	41(59%)	29 (41%)	
Age at diagnosis, mean $\pm$ SD	53.6 $\pm$ 16.9	56.4 $\pm$ 17.0	52.6 $\pm$ 16.2	0.34
Sex				0.95
Female	52 (58%)	23 (58%)	17 (59%)	
Male	37(42%)	17 (42%)	12 (41%)	
Unspecified	1	1	0	
Differentiation				0.25
Well	20 (25%)	6(17%)	7(25%)	
Moderate	41 (50%)	20 (57%)	14 (50%)	
Poor	20 (25%)	9 (26%)	7(25%)	
Unknown	9	6	1	
Site ^b				0.23
Proximal colon	24 (27%)	10 (25%)	11 (38%)	
Distal colon	20 (23%)	8 (20%)	7(24%)	
Rectum	45 (50%)	22 (55%)	11 (38%)	
Colon, unspecified	1	1	0	
Mucinous differentiation				
No	51 (73%)	21 (70%)	20 (80%)	0.43
Yes	10(14%)	5(17%)	2(8%)	
Focal	9(13%)	4(13%)	3(12%)	
Unknown	20	11	4	
Signet Ring cells				
No	59 (82%)	28 (88%)	22 (85%)	-
Yes	2 (3%)	1 (3%)	0	
Focal	11 (15%)	3 (9%)	4(15%)	
Unknown	18	7	3	
Crohn-like response				
No	18 (43%)	9 (39%)	6 (43%)	1.00
Yes	24 (57%)	14 (61%)	8 (57%)	
Unknown	48	18	15	
Mean number of TILs ^c , mean $\pm$ SD	4.4 $\pm$ 13.8	5.1 $\pm$ 16.9	3.2 $\pm$ 4.7	0.58
Growth pattern				
Expansive	31 (72%)	15 (63%)	13 (87%)	0.15
Invasive	9(21%)	7 (29%)	2(13%)	
Expansive and invasive	3(7%)	2 (8%)	0	
Unknown	47	23	14	
Astler-Coller stage				
A	5 (6%)	4(10%)	0	0.06
B1	11 (12%)	6(15%)	2 (7%)	

	All samples	MSS/MSI-Low	MSI-High	<i>p</i> -Value ^a
B2	24 (27%)	12 (30%)	11 (38%)	
C1	22 (25%)	11 (28%)	7 (24%)	
C2	12 (13%)	4(10%)	4(14%)	
D	15 (17%)	3 (7%)	5(17%)	
Not specified	1	1	0	
<i>KRAS</i> mutation	23/76 (26%)	9/41 (22%)	9/29 (31%)	0.12
<i>BRAF</i> mutation	0	0	0	-
Loss of MLH1 expression	4/42 (10%)	1/42 (2%)	3(7%)	
Loss of PMS2 expression	4/48 (8%)	1/48 (2%)	3 (6%)	-

^aComparison between MSS/MSI-Low and MSI-High.

^bProximal colon includes cecum, ascending colon, hepatic flexure, transverse colon; distal colon includes descending colon and sigmoid).

^cTumor-infiltrating lymphocytes.

Table 2

Frequency of microsatellite instable (MSI-High) tumors in African Americans and Native Africans from several studies.

	Ghana ^a	African Americans [9]	African Americans [10]	African Americans [12]	African Americans [11]	African Americans [8]
MSS	27 (39%)	11(50%)	27 (53%)	178 (81.2%) ^b	66 (69%) ^b	170 (85%) ^b
MSI-Low	14 (20%)	1 (5%)	2(4%)			
MSI-High	29 (41%)	10 (45%)	22 (43%)	44 (19.8%)	29 (31%)	29 (15%)

^aResults of the present study.

^bThese studies presented combined MSS and MSI-Low numbers.