COLORECTAL CANCER PATHOLOGY AT MUHIMBILI NATIONAL HOSPITAL, DAR ES SALAAM, TANZANIA

By

Gervais Ntakirutimana

A Dissertation Submitted in partial Fulfillment of the Requirements for the Degree of Master of Medicine (Anatomical Pathology) of Muhimbili University of Health and Allied Sciences

Muhimbili University of Health and Allied Sciences
October, 2016
CERTIFICATION

The undersigned certifies that he has read and hereby recommends for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled: “Colorectal Cancer Pathology at Muhimbili National Hospital, Dar es Salaam, Tanzania”, in partial fulfillment of the requirements for the degree of Master of (Anatomical Pathology) of Muhimbili University of Health and Allied Sciences.

___________________________________
Professor James Kitinya
(Supervisor)

___________________________________
Date
DECLARATION AND COPYRIGHT

I, Gervais Ntakirutimana, declare that this dissertation is my own original work and that it has not been presented and will not be presented to any other university for a similar or any other degree award.

Signature……………………………… Date………………………………

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Finally but not least, I am indebted to all pathologists, laboratory technicians, scientists and other house staff in the department of pathology, they made a smooth and lovely atmosphere to make my work a success.
DEDICATION

To Almighty God

To my beloved mother

To my family

To my wife ICYIMPAYE Devothe

To my daughter Amina Teth Devaritha

To all my teachers

To all my friends and colleagues

I dedicate this work.
ABSTRACT

Background: Colorectal cancer (CRC) is among the most common cancers worldwide especially in the Western world. An increase of CRC in resource-limited countries has been noted with presentation in the younger age group and advanced stages. It was therefore against this background that we sought to describe the clinicopathological characteristics of CRC at Muhimbili National Hospital in Tanzania.

Objectives: Our broad objective was to describe the clinicopathological features of CRC at Muhimbili National Hospital (MNH).

Methodology: The study was conducted in pathology department, histopathology unit at MNH, Dar es salaam, Tanzania. It was a descriptive cross-sectional study. Paraffin embedded blocks of colorectal cancer patients diagnosed between January 2011 and December 2014, were retrieved in departmental archive, resectioned, and stained by routine H&E and immunohistochemistry. More information pertaining to study participants was retrieved in hospital medical records. Microscopic examination was done for typing, grading, pathological staging using TNM-7 system and Dukes’ system.

Results: A total of 201 colorectal cancer patients were enrolled in our study, males slightly outnumbered females, with sex ratio of 1.1:1, mean age was 50.2 (SD±16.9), age range of 12-92, with a peak in 45-59 year age group and mode of 45 years. The rectum was the most commonly involved site (63.7%), usual adenocarcinoma was the most common histologic type (77.6%). Sixty one (30%) patients presented with high grade cancer and 88.2 % had advanced stages. Mucinous adenocarcinoma and signet ring cell carcinoma were found to correlate with young age (below 40 years), p=0.002. HIV/AIDS was the most common comorbidity associated with CRC (38%).

Conclusion: Colorectal cancer is not rare in Tanzania; poor prognostic entities are relatively common at younger age associated with advanced stage at diagnosis. There is a need for initiating a country wide screening program and through improved awareness and early detection and diagnosis it will enable curative interventions of CRC.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>Cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CUHAS</td>
<td>Catholic University of Health and Allied Sciences</td>
</tr>
<tr>
<td>DMP</td>
<td>Deep muscular plexus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary non polyposis colorectal cancer</td>
</tr>
<tr>
<td>ICC</td>
<td>Interstitial cell of Cajal</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MNH</td>
<td>Muhimbili National Hospital</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor, Nodes, Metastasis</td>
</tr>
<tr>
<td>UICC</td>
<td>International Union Against Cancer</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>Wnt</td>
<td>wingless-related integration site</td>
</tr>
<tr>
<td>HPF</td>
<td>High Power Field of microscope</td>
</tr>
</tbody>
</table>
DEFINITION OF TERMS

Cancer staging: Is the process of determining the extent to which a cancer has developed in the body. Contemporary practice is to assign a number from I-IV to a cancer, with I being an isolated cancer and IV being a cancer which has spread to the limit of what the assessment measures. The stage generally takes into account the size of a tumor, whether it has invaded adjacent organs, how many regional (nearby) lymph nodes it has spread to (if any), and whether it has appeared in more distant locations (metastasized).

Tumor grade: Is the description of a tumor based on how abnormal the tumor cells and the tumor tissue look under a microscope. It is an indicator of how quickly a tumor is likely to grow and spread. If the cells of the tumor and the organization of the tumor’s tissue are close to those of normal cells and tissue, the tumor is called “well-differentiated.” These tumors tend to grow and spread at a slower rate than tumors that are “undifferentiated” or “poorly differentiated,” which have abnormal-looking cells and may lack normal tissue structures. The latter generally have a more aggressive, or rapidly evolving biological behavior.

Hematoxylin: Is a natural substance that itself has no staining properties, not until it has been oxidized to hematein and combined with a mordant (most commonly aluminum alum). It is a positively charged (cationic) basic dye that stains nuclei in histological sections blue.

Eosin: Is a negatively charged (anionic) acid dye, which stains cytoplasm and extracellular matrix pink in histological sections (51).

Right hemicolon: Segment of the colon from the cecum, ascending colon, hepatic flexure or transverse colon.

Left hemicolon: Segment of the colon from splenic flexure, descending colon or sigmoid colon and rectum (37).
INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

In 2008 cancer was the leading cause of mortality worldwide, responsible for the deaths of an estimated 7.6 million people (1). Colorectal cancer (CRC) accounted for over 600 000 of those deaths, with low– and middle–income countries being the epicenter of the crisis accounting for 70 % of these deaths. There is reported variability in the clinicopathologic patterns of CRC in different geographical regions, for instance the mortality rate is higher in Central and Eastern Europe (20.3 per 100000 in men and 12.1 per 100000 for female), than in Central Africa, Congo (Brazzaville) (3.5 and 2.7, respectively) (2,3).

These geographic differences appear to be attributable to differences in dietary and environmental exposures that are peculiar upon a background of genetically susceptible individuals. However, despite this variation, the molecular characteristics keep on being similar throughout the world. In developed countries there has been a tremendous decrease in mortality rate that could be attributable to screening programmes especially in patients above 50 years, a reverse trend is being reported in several developing countries especially in young age groups. There are no screening programmes in all developing countries (2,4,5).

In Uganda, CRC increased from 3 to 6.8 in females, and from 2.7 to 6.6 per 100,000 in males over the period 1960-1997. Topography of the tumor is also different with low risk areas having the caecum as the commonest site of the tumor, whereas in high risk areas the tumor commonly affects the left side of the colon (6).

In the United Republic of Tanzania, epidemiological data is scanty. Between 1975-1979, CRC was the sixteenth among the leading common cancers in males and fifteenth in females with the age standardized incidence rate of 1.6 per 100,000 and 2.1 per 100,000 respectively (Kilimanjaro cancer registry), between 1980-1981 it was seventh in males and third in females with prevalence rate of 3.8 per 100,000 and 3.6 per 100,000 respectively (Muhimbili Medical
Thus, the present investigation is aimed at evaluating clinicopathological features of CRC at Muhimbili National Hospital (MNH) from January 2011 through December 2014.

**Normal anatomy**

The large bowel is a tubular structure of 1–1.5 m of the gastrointestinal tract and is divided into following regions of varying dimensions: caecum measures 6 to 8 cm long and breadth this large size implies late obstructive signs associated with tumors at that site; ascending (right) colon measures 12 to 20 cm and covered by peritoneum anteriorly and on both sides, transverse colon is the longest portion of 40 to 50cm, it is a mobile portion which takes varying configurations according to the position of the person, descending (left) colon, sigmoid colon is a redundant portion of varying lengths, and rectum is 10cm long which follows the sacral curvature and ends at the anal canal. The hepatic flexure is at the junction of the ascending and transverse colon, and the splenic flexure is at the junction of the transverse and descending colon.

From caecum up to splenic flexure, the blood supply is by superior mesenteric artery and its branches; distal colon is supplied by the inferior mesenteric artery while the lower rectum is supplied by middle and inferior rectal arteries which are branches of internal iliac artery.

Lymphatic drainage of colon is to paracolic lymph nodes along with vascular arcades at different levels, up to the level of superior and inferior mesenteric artery. Drainage of the rectum is to lymph node chains along the inferior mesenteric artery, haemorrhoidal chain, hypogastric and common iliac nodes.

The large bowel like other parts of gastrointestinal tract is composed of: mucosa, submucosa, muscularis externa (propria), and serosa (in the rectum, perimuscular tissues). The mucosa (mucous membrane) has three components: epithelium, lamina propria which is a loose connective tissue, and muscularis mucosae. The mucosal surface is lined by columnar epithelium composed of absorptive cells and goblet cells synthesizing and secreting mucin granules. Crypts of Lieberkühn open into the surface epithelium mainly and they are lined by...
absorptive cells and goblet cells as well, but in addition they contain undifferentiated precursor cells, endocrine cells and Paneth cells containing lysozyme, epidermal growth factor and other substances (7,8).

Crypts are tubular, arranged parallel to each other in the mucosa. Any branching should exclude an ongoing inflammation. Paneth cells are normally found in caecum and proximal right colon, occurrence at another site is usually a metaplastic process of inflammatory origin (9).

The lamina propria is a loose connective tissue composed of few lymphocytes, plasma cells, histocytes, smooth muscles fibres, blood and lymphatic channels confined above by muscularis mucosa and nerves in a network of collagen fibers (9). Submucosae is a loose connective tissue containing same components like lamina propria and in addition contains neural plexus of Meissner (9).

Muscularis externa (propria) is composed of inner circular and outer longitudinal layer with myenteric neural plexus of Auerbach between them. The serosa is lined by a single flattened to cuboidal layer of mesothelial cells and subadjacent fibroelastic tissue (11).

Interstitial cells of Cajal (ICC) are widely dispersed within the submucosal (ICC-SM), intramuscular (ICC-IM, ICC-DMP) and inter-muscular layers (ICC-MY) of the gastrointestinal tract all the way from the esophagus to the internal anal sphincter (7,8,10). ICC operate as electrical pacemakers and generate spontaneous electrical slow waves which constitute the basic electrical rhythm in the gastrointestinal tract. Additionally, they are essential for the active propagation of slow waves and mediate neurotransmission between inhibitory and excitatory enteric neurons and smooth muscle cells. Loss or dysfunction of ICC networks has been linked with slow transit constipation, idiopathic megacolon, diabetic gastropathy, and other diseases of defective gastrointestinal motility. Typical ICC are characterized by their spindle-shaped, elongated body with several branches and processes. The nucleus is ovoid and a basal lamina and a thick capsula composed of collagen may envelope the cells (11).
Hitomi Maeda established an-to-use marker protein for ICC, identifying ICC as Kit-expressing cells using an anti-Kit antibody Maeda et al (11). The protein encoded by \textit{c-kit} is the receptor tyrosine kinase Kit (CD117) which is essential for development and function of ICC. Although the knowledge of the role of ICC in gastrointestinal disorders is evolving rapidly, no major breakthrough has been achieved in treatment so far. The Kit inhibitor Imatinib mesylate has been shown to be successful in Kit expressing tumors (GISTs), but no drugs improving loss of c-Kit function are available today. Hopefully new medications modulating gastrointestinal peristalsis may be provided in the future. Replacement of faulty pacemaker cells however, will be a prospective promise of genetic therapy at best (11).

**Colorectal carcinoma**

**Epidemiology**

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of death. It affects men and women almost equally. It is the third most common cancer and the third leading cause of cancer death in men and women in the United States (12).

Recently, its incidence has increased in most European and Asian countries, but in America, a reverse trend was observed (1). The peak age at presentation is 75+ years, reports of presentation at younger age (< 40 years) associated with advanced stage and grade of tumor have been made (13). The etiology of this deviation from usual presentation is unknown, but HIV immunosuppression may be one factor (3,10).

In Africa CRC is the fifth among common leading cancers in males with incidence rate of 6.9 cases /100,000 and occupies fourth rank in females with incidence rate of 5.0 cases/100,000 (13). Based on the available evidence, the incidence of CRC in SSA (Sub-Saharan Africa) appears to be much lower than in high–income countries. However, the trends associated with sex and ages were very similar with slight predilection in males compared to females. The incidence of colorectal cancer in Eastern Africa still high and substantially higher in males (4.5 cases /100,000) than in females (4.1 cases/100,000)(15). Epidemiological data in
Tanzanian population is scanty with the prevalence rate of 3.8 per 100,000 published in 1980s but nationwide data is not yet available.

Surgery is the best treatment option among several treatment modalities such as chemotherapy, radiotherapy and molecular based targeted therapies, despite that heterogeneity of treatment, colorectal cancers continue to emerge in current era with different histological types, a review of pathogenesis and genetic aspects are needed to try to highlight eventual causes of such diversity (16).

**Pathogenesis**

Data has shown that 50% of US the population develop adenomatous polyps by age 70, but only one tenth will progress to cancer (12,13). Evidence (both laboratory and epidemiological) showed that colorectal cancer is a progressive, multistep genetic alterations supported by environmental factors (14,15,16). Some individuals have been shown to be prone to the development of CRC and a familial tendency was found where a number of patients with CRC had first or second degree relatives with CRC.

It has been reported that 5% of colon cancers may due to single gene syndromes like familial adenomatous polyposis (FAP). Lynch syndrome is also referred to as hereditary non polyposis colorectal cancer (HNPCC) (17,18). Mutations associated with these syndromes have been elucidated. FAP is caused by germline mutation of the tumor suppressor gene, adenomatous polyposis coli (APC) located on locus 5q (5q21).

In addition, somatic or acquired alterations (80 to 90% of cases) of APC are responsible for familial and sporadic colorectal cancers (12,19). Lynch syndrome is caused by mutations in genes involved in mismatch DNA repair such as *hMLH1 and hMSH2*. Table1 below depicts hereditary syndromes and respective genes involved in the development of colorectal cancer.
Table 1: Hereditary syndromes predisposing to colorectal cancer (12,20).

<table>
<thead>
<tr>
<th>Strongly predisposed to cancer</th>
<th>Responsible genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAP</td>
<td>APC (dominant), c- myc (recessive)</td>
</tr>
<tr>
<td>Lynch syndrome (HNPCC)</td>
<td>hMLH1, hMSH2, hMSH6, PMS1, PMS2, hMLH3, EXO1</td>
</tr>
<tr>
<td>Weakly predisposed to cancer</td>
<td></td>
</tr>
<tr>
<td>Peutz-jeghers syndrome</td>
<td>STK11 (or LKB1)</td>
</tr>
<tr>
<td>Juvenile polyposis</td>
<td>SMAD4/MADH4 or BMPR1A</td>
</tr>
<tr>
<td>Cowden disease</td>
<td>PTEN/MMAC1</td>
</tr>
<tr>
<td>Bannayan-Roalccaba syndrome</td>
<td>PTEN</td>
</tr>
<tr>
<td>Li-Fraumeni syndrome</td>
<td>p53</td>
</tr>
<tr>
<td>Bloom syndrome</td>
<td>Blm</td>
</tr>
</tbody>
</table>

**Molecular pathogenesis**

APC protein interacts with an intracellular protein called β-catenin, which once activated will be translocated into the nucleus where it increases cellular proliferation by transcriptional activation of c-myc, cyclin D1 and peroxisome-proliferator-activated receptor delta (PPARσ). β-catenin protein is also a component of intercellular adhesion complex that maintains homotypic cell adhesion, once its concentration raises, the cell proliferation program is activated (16). In concert with other factors, APC protein arrests cell proliferation and causes apoptosis by phosphorylating β-catenin which promotes its ubiquitination hence its degradation through proteosome pathway (16).

So, any inactivating mutation in APC locus will abolish APC–mediated β-catenin degradation, and its nuclear concentration remains high which will lead to the formation of an adenoma. Any additional mutation, for instance inactivation of p53, will favour the progression of adenoma to adenocarcinoma (12,21).
Chromosomal abnormalities

Sporadic CRC is a genetic disease caused by sequencial accumulations of mutations in multiple genes. Molecular evidence has elucidated crucial genes that predispose to the development of sporadic CRC, and three pathways are implicated in this progression: chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP). Most of sporadic CRC evolve from aberrations in the CIN pathway which is the adenoma –carcinoma pathway. In the latter pathway, CRC results from mutational activation of oncogenes (K-ras, C-Src, C-myc) and inactivation of tumor suppressor genes (APC, TP53, heterozygozity of the long arm of chromosome 18;18q LOH ) (22, 19, 23, 24). Specific chromosomal loci have been identified with high frequencies of allelic losses such as chromosomes 17p, 18q and 5q (19, 22).

Genes like p53 is located on 17p, in 75% of tumours there is a LOH at 17p locus and p53 gene is inactivated by mutation. Allelic deletion of APC gene on chromosome 5q was observed in 50% of cases associated with adenomas and adenocarcinomas (23, 24).

Other suppressor genes like, deleted in colorectal carcinoma gene (DCC), SMAD-4, SMAD-2 both located on chromosome 18q once inactivated are independent prognostic factors of CRC. Activation of oncogene K-ras on chromosome 12, which encodes proteins involved in transmission of extracellular growth signals into nucleus, induces missense mutation favouring continuous growth signals (16). The figure 1 below summarizes chromosomal and molecular events underlying the pathogenesis of colorectal cancer.
Figure 1: Pathways and genetic targets of colorectal cancer carcinogenesis

Initiation of CRC carcinogenesis seems to involve, wnt (wingless-related integration site) signaling pathway which is disrupted by either mutations or deletions of the APC gene or by mutations in the β-catenin gene.

Tumor formation can go on through the CIN pathway, characterized by multiple allelic losses of tumour-suppressor genes and mutations of the oncogene, k-ras (14, 25, 26, 27). Alternatively, mutations of MMR genes direct to the MSI phenotype as in the Lynch syndrome or with acquired inactivation of the hMLH1 gene (28,29,32). Another mechanism of colorectal carcinogenesis takes place through the CpG island methylator phenotype (CIMP), which silences genes through promoter methylation. CIMP can proceed through silencing the hMLH1 gene causing the MSI phenotype (CIMP+/MSI-). Alternatively, a diversity of tumour-suppressor genes other than hMLH1 can be silenced through promoter methylation (CIMP+/MSI-,
or CIMP /MSI-L). Most likely new genes responsible in CRC heterogeneity will be discovered (31,32).

**Clinical presentation**
Clinical presentation depends on the site and size of the tumor. By virtue that tumors of the right side of the colon (ceacum and ascending colon) grow exophytically, it is rare for these tumors to present with signs of obstruction, rather they present with rectal bleeding and signs of anemia associated with ulceration. On the other hand tumors arising on the left side of the colon, especially rectosigmoid, are flat and infiltrating and they tend to be circumferential and obstruct the lumen of the bowel like napkin ring (37). So in the later case intestinal obstruction will be the main presenting feature.

**Clinical stage**
Clinical stage at diagnosis determines extent of disease hence the prognosis and best outcomes are expected in early stages, which is evaluated using tumor-nodes-metastasis (TNM) of International Union Against Cancer (33,34,35). Imaging techniques are used to determine the extent of the tumor, lymph node status and eventual metastasis at the time of diagnosis. It can be supplemented by pathological stage which is considered more accurate after surgical resection (40). This is then used to determine the stage of the disease.

**Histological classification**
For consistency and uniformity of classification of colorectal cancer the following tables depict colorectal tumours as adapted from World Health Organization Classification of Tumours Pathology and Genetics of Tumours of the Digestive System, 2010, 4th edition (52).
<table>
<thead>
<tr>
<th>Epithelial tumours</th>
<th>Serrated lesions</th>
<th>Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoma, NOS</td>
<td>Sessile serrated adenoma/polyp</td>
<td>Andenocarcinoma ,NOS</td>
</tr>
<tr>
<td>Tubular adenoma, NOS</td>
<td>Serrated polyposis</td>
<td>Cribriform comedo-type adenocarcinoma</td>
</tr>
<tr>
<td>Villous adenoma, NOS</td>
<td>Traditional serrated adenoma</td>
<td>Medullary carcinoma, NOS</td>
</tr>
<tr>
<td>Tubulovillous adenoma, NOS</td>
<td></td>
<td>Micropapillary carcinoma</td>
</tr>
<tr>
<td>Glandular intraepithelial neoplasia, low grade</td>
<td></td>
<td>Mucinous carcinoma</td>
</tr>
<tr>
<td>Glandular intraepithelial neoplasia, high grade</td>
<td></td>
<td>Serrated adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Signet ring cell carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenosquamous carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spindle cell carcinoma, NOS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Squamous cell carcinoma, NOS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Undifferentiated carcinoma</td>
</tr>
</tbody>
</table>
Histological classification of colorectal tumours (continued)

<table>
<thead>
<tr>
<th>Carcinomas (continued)</th>
<th>Non-epithelial tumours</th>
<th>Malignant lymphomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroendocrine neoplasms</td>
<td>Mesenchymal tumors</td>
<td></td>
</tr>
<tr>
<td>Neuroendocrine tumor G1 (NET G1) / Carcinoid</td>
<td>Leiomyoma, NOS</td>
<td>Extramedullary marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)</td>
</tr>
<tr>
<td>Neuroendocrine tumor G2 (NET G2)</td>
<td>Lipoma, NOS</td>
<td>Mantle cell lymphoma</td>
</tr>
<tr>
<td>Neuroendocrine carcinoma, NOS</td>
<td>Angiosarcoma</td>
<td>Diffuse large B-cell lymphoma (DLBCL), NOS</td>
</tr>
<tr>
<td>Large cell neuroendocrine carcinoma</td>
<td>Gastrointestinal stromal tumor, malignant</td>
<td>Burkitt lymphoma, NOS</td>
</tr>
<tr>
<td>Small cell neuroendocrine carcinoma</td>
<td>Kaposi sarcoma</td>
<td>B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma</td>
</tr>
<tr>
<td>Mixed adenoneuroendocrine carcinoma</td>
<td>Leiomyosarcoma, NOS</td>
<td></td>
</tr>
<tr>
<td>L cell, Glucagon-like peptide-producing and PP/PYY-producing NETs</td>
<td>Perineurioma, NOS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ganglioneuroma</td>
<td></td>
</tr>
</tbody>
</table>
For most histological types, no stage is of independent prognostic type other than from small cell carcinoma, signet ring cell carcinoma which have poor prognosis and medullary carcinoma which has good prognosis (36,37).

**Grading**

Tumor grading has been an issue of subjectivity where strata are formed based on one histological criteria: For example, degree of gland formation. Despite that, it was revealed to be stage –independent in prognostification of patients with CRC (36, 38, 39). Irrespective of the complexity of criteria used by different methods, most classify CRC into four grades:

Grade 1: Well differentiated

Grade 2: Moderately differentiated

Grade 3: Poorly differentiated

Grade 4: Undifferentiated

**Pathological staging**

It is the best estimate of prognosis since it determines the anatomical extent of the disease. TNM staging of AJCC and International Union Against Cancer is recommended (40,41,42,43). In this system “T” refers to the extent of the tumor at the primary site at the time of diagnosis, before any treatment. ”N” refers to the status of regional lymph node and ”M” refers to the distant metastatic disease as well as nonregional lymph nodes. Symbol” p” used as a prefix refers to pathological determination of TNM as opposed to clinical determination designed by a prefix” c”. The following paragraphs explain different TNM definitions and groups.
**Table 3: AJCC/UICC TNM-7 definitions (42,43).**

<table>
<thead>
<tr>
<th>Primary tumor (T)</th>
<th>Regional lymph nodes (N)</th>
<th>Distant Metastasis (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tx</strong>: Primary tumor cannot be assessed</td>
<td><strong>NX</strong>: Regional lymph nodes cannot be assessed</td>
<td><strong>MX</strong>: Presence of distant metastasis cannot be assessed</td>
</tr>
<tr>
<td><strong>T0</strong>: No evidence of primary tumor</td>
<td><strong>N0</strong>: No regional lymph node metastasis</td>
<td><strong>M0</strong>: No distant metastasis</td>
</tr>
<tr>
<td><strong>Tis</strong>: Carcinoma in situ (intraepithelial carcinoma)</td>
<td><strong>N1a</strong>: Metastasis in one regional node and <strong>N1b</strong>: (Metastasis in two or three nodes)</td>
<td><strong>M1</strong>: Distant metastasis</td>
</tr>
<tr>
<td><strong>T1</strong>: Tumor invades the submucosa</td>
<td><strong>N2</strong>: Metastasis in 4 or more lymph nodes</td>
<td><strong>M1a</strong>: For a single metastatic site</td>
</tr>
<tr>
<td><strong>T2</strong>: Tumor invades the muscularis propria</td>
<td><strong>N2a</strong>: Metastasis in four to six nodes</td>
<td><strong>M1b</strong>: For multiple metastatic sites</td>
</tr>
<tr>
<td><strong>T3</strong>: Tumor invades through the muscularis propria into the subserosa or into the non peritonised pericolic or perirectal tissues.</td>
<td><strong>N2b</strong>: Metastasis in seven or more nodes</td>
<td></td>
</tr>
<tr>
<td><strong>T4</strong>: Tumor directly invades other organs or structures</td>
<td><strong>T4a</strong>: The tumour penetrates the surface of the visceral peritoneum</td>
<td><strong>T4b</strong>: The tumour directly invades other organs or structures</td>
</tr>
</tbody>
</table>

NB: T1-2 lesions that lack regional lymph node metastasis but have tumour deposits are classified as **N1c**.
**Table 4: TNM-7 Stage groups, 2010 (47).**

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Dukes</th>
<th>MAC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
<td>A</td>
<td>B1</td>
</tr>
<tr>
<td>II A</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
<td>B</td>
<td>B2</td>
</tr>
<tr>
<td>IIB</td>
<td>T4a</td>
<td>N0</td>
<td>M0</td>
<td>B</td>
<td>B2</td>
</tr>
<tr>
<td>IIC</td>
<td>T4b</td>
<td>N0</td>
<td>M0</td>
<td>B</td>
<td>B3</td>
</tr>
<tr>
<td>III A</td>
<td>T3-T4a</td>
<td>N1/N1c</td>
<td>M0</td>
<td>C</td>
<td>C1</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N2a</td>
<td>M0</td>
<td>C</td>
<td>C1</td>
</tr>
<tr>
<td>IIB</td>
<td>T3-T4a</td>
<td>N1/N1c</td>
<td>M0</td>
<td>C</td>
<td>C2</td>
</tr>
<tr>
<td></td>
<td>T2-T3</td>
<td>N2a</td>
<td>M0</td>
<td>C</td>
<td>C1/C2</td>
</tr>
<tr>
<td></td>
<td>T1-T2</td>
<td>N2b</td>
<td>M0</td>
<td>C</td>
<td>C1</td>
</tr>
<tr>
<td>IIC</td>
<td>T4a</td>
<td>N2a</td>
<td>M0</td>
<td>C</td>
<td>C2</td>
</tr>
<tr>
<td></td>
<td>T3-T4a</td>
<td>N2b</td>
<td>M0</td>
<td>C</td>
<td>C2</td>
</tr>
<tr>
<td></td>
<td>T4b</td>
<td>N1-N2</td>
<td>M0</td>
<td>C</td>
<td>C3</td>
</tr>
<tr>
<td>IV A</td>
<td>Any T</td>
<td>Any N</td>
<td>M1a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IVB</td>
<td>Any T</td>
<td>Any N</td>
<td>M1b</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

MAC: Modified Asler-Coller stage, 1954
**DUKES staging**

In 1932, DUKES devised a method which has been famous and proved to predict the prognosis, by this staging we have following categories: (38, 42).

Dukes’ A: Growth confined to the wall of the bowel  
Dukes’B: Growth reaching the serosa and beyond, but no nodal involvement  
Dukes’ C: Growth beyond serosa and involving regional lymph nodes  
Dukes’D: Metastatic disease.

**Conceptual framework**

Clinical data on CRC  
Pathological data on CRC

![Figure 2: Conceptual framework](image)

**Figure 2: Conceptual framework.** This is a diagram explaining the key concept and different variables that were analyzed in this study.
PROBLEM STATEMENT AND RATIONALE

Problem statement
Recently, CRC has shown a change in trend and presentation both clinically and histologically. It is increasingly seen among younger age groups in the black population especially in resource-limited countries like Tanzania. For example, time trend studies show an increase in incidence of CRC of 3 cases per 100,000 population between 1960-1966 and 8.3 cases per 100,000 population between 1991-1994 in the Ugandan population. Affected patients present with high grade tumors at advanced stage, thus poor prognosis. However, data on the association of specific CRC subtypes and their risk factors including age remain scanty (45,46).

This study was therefore conceptualized to describe the clinicopathological features of CRC seen in patients at MNH to establish whether there is a similar trend in this population as seen in the others.

Study rationale
Colorectal cancer is one of malignancies whereby surgery is the treatment of choice especially when it is in early stages. Unfortunately, most patients present late hence poor prognosis. Conclusions drawn from this study will be of help for both health care providers and police makers aiming at early therapeutic interventions and establishing a screening program in certain age group of predicted bad outlook.

Research question
Is there any association between CRC type, grade, stage at presentation and young age group in patients seen at MNH?

Hypothesis
CRC presents with a high histological grade and at a younger age in Tanzania.
Objectives

Broad objective
To describe the clinicopathological features of CRC in patients presenting at MNH.

Specific objectives

1. To determine different histological types of CRC diagnosed at MNH from January 2011 to December 2014.
2. To determine grades and pathological stages of different types of CRC.
3. To compare the frequency of CRC grade in different age groups.
4. To determine the association of colorectal cancer with other factors (gender, subsite, and comorbidities).
MATERIALS AND METHODS

Study setting
The study was conducted at Muhimbili National Hospital (MNH) which is the national, referral and university teaching hospital based in Dar es Salaam on the shores of Indian Ocean in the United Republic of Tanzania. The histopathology unit at the CPL (central pathology laboratory) performs routine staining, histochemistry, immunohistochemistry and other diagnostic procedures on patient biopsies; and has a system of archiving that allowed us to retrieve tissue blocks and surgical pathology reports of patients targeted by our investigation. MNH medical records department was consulted to get important information not provided in laboratory request forms.

Study design
This was a cross-sectional, descriptive laboratory study.

Study population
All patients who underwent endoscopic biopsy, radical surgery for colorectal cancer and surgical specimens submitted for histology evaluation from January 2011 to December 2014

Inclusion criteria
Every participant who was diagnosed clinically and histologically to have CRC

Exclusion criteria
Patients who have undergone colorectal surgery for other reasons other than CRC were excluded from the study. Those whose tissue blocks were missing were excluded as well.

Sampling

Sampling procedure
Through archives and hospital medical records department, demographics and important clinical data were recorded in a data sheet; corresponding blocks were resection and stained by routine H &E, special stains and immunohistochemistry techniques.
Estimation of the sample size

The sample size was calculated based on known frequency of colorectal cancer found at Catholic University of Health and Allied Sciences Bugando (CUHAS - Bugando) based in Western part of Tanzania (p = 4.7%) (37).

\[
n = \frac{z^2 \cdot p(1-p)}{\varepsilon^2}
\]

\( z \): z value (e.g. 1.96 for 95% confidence level)

\( p \): prevalence (4.7%)

\( \varepsilon \): standard error: 0.03

\[
n = \frac{4 \cdot 0.047 \cdot 0.953}{0.0009} \approx 200
\]

Laboratory methods

Hematoxylin and Eosin stain

Microtomy of 4µ thick sections was performed; after which sections were de-waxed by placing the slides on a hot plate and microwaved in the oven at 60°C for 50 minutes, before being placed in xylene. Thereafter, a routine H&E stain was done, followed by clearing in xylene and mounting as detailed in appendix II (51).

Periodic acid Schiff (PAS) histochemical method

After making 4micron thick paraffin sections, deparaffinization was done on hot plate then hydration using descending concentration of alcohol to distilled water, followed by placing slides consecutively into 0.5% periodic acid, distilled water, microwaved Schiff’s reagent, washing in running tap water. Counterstaining was done using filtered Mayer’s hematoxylin, washing in tap water, then dehydration, clearing cover slipped using DPX (dibutyl phthalate xylene). Positivity was assessed by looking at cytoplasmic magenta color and a kidney section was used as positive control (51). See details in appendix IV.
Reticulin histochemical method
As for other methods above, following procedures were done, 4 micron thick sections, deparaffinization, oxidation in acidified potassium permanganate, decolorization with oxalic acid, mordanting in iron alum, impregnation in ammoniacal silver solution, Toning in gold chloride, counterstained with neutral red. Lastly, dehydration, clearing and mounting were done. Positivity was evaluated by black color with red nuclei. Normal liver was used as positive control (51). Details are found in appendix III.

Immunoperoxidase staining method: See details in appendix V
Immunoperoxidase technique was used as previously described (51,67) on tissue sections, mounted on SuperFrost® slides (Menzel GmbH & Co KG, Braunschweig, Germany) deparaffinized, rehydrated and boiled in a microwave in citrate buffer at pH 9 for antigen retrieval. Quenching of endogenous peroxidase activity, was done by incubating the sections in hydrogen peroxide in distilled water at room temperature, washing in Tris-buffered saline (TBS). The sections were then incubated with the respective primary antibody including, mouse monoclonal to pan-leucocyte anti-human CD45(clone LCA), B-cell anti-human CD20 (clone L26), pancytokeratin (AE1/AE3), which is a prediluted antibody. All anti-mouse antibodies from DakoCytomation, (Glostrup, Denmark). Subsequently, the sections were rinsed with Tris buffer solution and developed (visualized) with Sigma DAB chromogen (Sigma-Aldrich, St. Louis MO, USA) and after TBS washing, lightly counter-stained with Harris Hematoxylin, blued in warm water, dehydrated in ascending grades of ethanol, cleared in two runs of xylene and mounted with DPX and coverslipped. Negative controls included sections from tissues not expressing the respective antigen as well as substitution of the primary antibody by buffer. Positive controls included tissue sections known to express the antigen under investigation (tonsils) for CD45, CD 20 and skin for pancytokeratin (67).

Positivity grading was obtained by determining the proportion of tumor cells with a cytoplasmic and membranous brown yellowish stain in 5 HPF. The table 5 below reflects primary antibodies used.
Table 5: Primary antibodies used for the study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Source</th>
<th>Antigen retrieval</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan cytokeratin</td>
<td>AE1/AE3</td>
<td>Dako, mouse monoclonal</td>
<td>EDTA 750 W</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>CD45</td>
<td>LCA</td>
<td>Dako, mouse polyclonal</td>
<td>EDTA 750 W</td>
<td>1:100</td>
</tr>
<tr>
<td>CD20</td>
<td>L26</td>
<td>Dako, mouse monoclonal</td>
<td>EDTA 750 W</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Microscopy

After above procedures, using light microscope OLYMPUS CX41, pictures were taken by an OLYMPUS SC30 camera and visualized by using OLYMPUS soft imaging solutions GMBH software in OSIS USB camera input mode. All epithelial tumors were graded as well differentiated (G1) if more than 95% of the tumor had gland formation, moderately differentiated (G2) if 50 to 90% with gland formation, poorly differentiated (G3) if more than zero up to 49% with gland formation, and undifferentiated (G4) if there was no gland formation, presence of squamous or sarcomatoid differentiation and these were assigned a numerical grade I, II, III and IV respectively. For easy analysis grade I and grade II were classified as low grade whereas grade III and grade IV were classified as high grade (52).

Other microscopic variables like nuclear pleomorphism, mitotic count per 10 high power fields were used to estimate the grade of both epithelial and non-epithelial tumors. Extent of invasion of the tumor within mucosa, submucosa, muscularis propria, serosa and regional lymph nodes reflected the stage. Our patients whose resection surgery was done, were staged according to the seventh edition of the International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) tumor staging system, Tumor-Node-Metastases (TNM) in stage I through IV, and modified Dukes staging system, in Dukes A through D. We omitted M status and pTN was assessed as per current pathological stage guidelines.
TNM stage I and II as well as Dukes A and B were classified as early stages, on the other hand TNM stage III and IV as well as Dukes C and D were assigned to an advanced stage.

**Data collection**
Every participant was given a unique identification code, relevant clinical information was retrieved in departmental records and missing data was retrieved using hospital record. The preforma sheet was used to gather all clinical and histomorphological data. The latter was described by the investigator and reviewed by the supervising senior pathologist.

**Statistical analysis**
Data entry and analysis were done using SPSS computer software version 20.0 (IBM Corp. Armonk, NY, USA). Data were summarized in the form of proportions and frequency tables for categorical variables. Continuous variables were summarized using mean, median, mode and standard deviation. Chi-square test was used to test for significance of associations between the predictor and outcome variables in the categorical variables. Categorical variables with data less than 5, Fisher exact test was used. Statistical significance was defined as a P value of <0.05.

**Limitations of the study**
During our study, we encountered a number of limitations to be pointed out like missing data and financial constraints among others, for instance HIV status was not taken for every patient with CRC especially those in the younger age group and our budget did not allow us to conduct enough immunohistochemical methods or advanced molecular methods, some of which are not available in our setting to ascertain HIV status. Hence we could not ascertain any correlation between variables like HIV and CRC. This study is hospital-based in the national referral hospital; it may not necessarily reflect the real picture of CRC in Tanzanian population.
**Ethical considerations**

Before the commencement of the study, permission was granted by the university ethical clearance committee, MNH management for access to archival data necessary for our study and by Head of department of pathology for archival data access. Patients’ data was treated anonymously, all information was kept with strict confidentiality and exempt of informed consent was requested from institutional review board (IRB).
RESULTS
During period of the study CRC accounted for 201 biopsies (5%) which constituted our study population, and 3771 biopsies (95%) were other malignant conditions from different parts of the body.

Patient demographics
There were slightly more men (n=105) than women (n=96) in our study population P=0.762, colorectal cancer peaked in the 45-59 year age group (mode 45 years) (Figure 3). Male: Female ratio was 1.1:1 and 28% of the patients were less than 40 years. The median age was 52 years; the youngest patient was 12 years while the oldest was 92 years with a mean age of 50.19 (SD-16.9). Table 6.

Tumor site
CRC was more common in the rectum 128 patients (63.7%), followed by right hemicolon 36 patients (17.9%), and left hemicolon 26 patients (12.9%), then rectosigmoid colon 11 patients (5.5%) Table 6.

Table 6: Clinicopathological characteristics of patients with CRC

<table>
<thead>
<tr>
<th></th>
<th>n= 201</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>105</td>
<td>52.2</td>
</tr>
<tr>
<td>Women</td>
<td>96</td>
<td>47.8</td>
</tr>
<tr>
<td>Patient age, years (median)</td>
<td>52( 12-92)</td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right hemicolon</td>
<td>36</td>
<td>17.9</td>
</tr>
<tr>
<td>Left hemicolon</td>
<td>26</td>
<td>12.9</td>
</tr>
<tr>
<td>Rectosigmoid colon</td>
<td>11</td>
<td>5.5</td>
</tr>
<tr>
<td>Rectum</td>
<td>128</td>
<td>63.7</td>
</tr>
</tbody>
</table>
Figure 3: Age distribution of male and female patients with CRC

Histological types of CRC
Microscopically, usual (conventional) adenocarcinoma was the most common histopathological tumor, seen in 154 (76.6%) patients, of these 36 (23%) patients were villoglandular adenocarcinoma coexisting with adenoma, followed by mucinous adenocarcinoma in 29 (14.4%) patients, signet ring cell adenocarcinoma in 9 (4.5%) patients, carcinoid tumor in 2 (1%) patients, gastrointestinal stromal tumor (GIST) in 2 (0.9%) which were confirmed with c-kit by previous independent study, diffuse large B cell lymphoma, squamous cell carcinoma and medullary carcinoma (not shown) in 1 (0.5%) patient each. See Figure 4 below.
Figure 4: Histological types of colorectal cancer patients at MNH
Table 7. Age and histological types of patients with colorectal cancer

<table>
<thead>
<tr>
<th>Type of colorectal cancer</th>
<th>Below 40 years</th>
<th>Above 40 years</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percent</td>
<td>Number</td>
</tr>
<tr>
<td>Usual adenocarcinoma</td>
<td>34</td>
<td>60.7</td>
<td>122</td>
</tr>
<tr>
<td>Mucinous adenocarcinoma</td>
<td>13</td>
<td>23.2</td>
<td>16</td>
</tr>
<tr>
<td>Signet ring cell adenocarcinoma</td>
<td>7</td>
<td>12.5</td>
<td>2</td>
</tr>
<tr>
<td>Carcinoid tumor</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Malignant Lymphoma</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>1</td>
<td>1.7</td>
<td>0</td>
</tr>
<tr>
<td>Undifferentiated carcinoma</td>
<td>1</td>
<td>1.7</td>
<td>1</td>
</tr>
<tr>
<td>Medullary carcinoma</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>56</strong></td>
<td><strong>100</strong></td>
<td><strong>145</strong></td>
</tr>
</tbody>
</table>

p=0.002

Mucinous adenocarcinoma 13 (23.2%) and signet ring cell adenocarcinoma 7 (12.5%) were more frequent in the young (below 40 years) in comparison to older age group (above 40 years) 16 (11%) and 2 (1.3%) respectively, and this difference was statistically significant, p=0.002.

There is no correlation between anatomical site and histological type, p=0.068.
Fifty five percent of mucinous adenocarcinomas and 55.5% of signet ring cell adenocarcinomas were located in the rectum followed by right hemicolon as it was found in other colorectal cancers.
Figure 5: Photomicrographs of histological types of CRC

5.1. Coexistence of normal mucosa (Middle field), tubular adenoma (Left field) and carcinoma (Right field) showed with an arrow (×200)
5.4. Signet ring carcinoma infiltrating lymph node, arrow depicts signet ring cell (×200).

5.5. Diffuse large B cell lymphoma (×100), showing diffuse growth pattern

5.6. High power of 5.5 showing large, oval cells, vesicular chromatin, more than one nucleoli (×200).

5.7. Immunohistochemistry of 5.6 showing negative staining with pancytokeratin (×200).
5.8. Histochemical staining with periodic acid Schiff showing negative staining of 5.6 (×200).

5.9. Positive reticulin consistent with lymphoid stroma of 5.8 (×200).

5.10. Strong membranous CD20 positivity of 5.9 confirming B cell lineage (×200).

5.11. Negative CD45 for undifferentiated carcinoma highlighting circumscribed solid sheets (×100).
5.12. Strong cytoplasmic pancytokeratin positive (x 200).

5.13. Epithelioid GIST with membranous and cytoplasmic c-KIT positive. Courtesy Dr. HATEGEKIMANA Claudien, Butare university teaching hospital, Rwanda.

5.14. Medullary carcinoma (x100) showing solid sheets

5.15. High power (x400) of 5.7, showing expansile border, lymphocytic infiltrate, perineural invasion (arrow), vesicular nuclei and moderate eosinophilic cytoplasm (inset).
Grading
The majority were moderately differentiated adenocarcinoma, which was found in 124 (62.3%), 59 (29.7%) were poorly differentiated adenocarcinoma and 14 (7%) were well differentiated adenocarcinoma. Two cases (1%) were anaplastic (undifferentiated) tumors. Figure 7.

Figur 7: Grades of colorectal adenocarcinomas

In general 69.3% of our population presented with a low grade CRC and 30.7% with high grade CRC that had an aggressive clinical behavior.

Staging
Pathological staging was carried out on 68 patients who underwent resection surgery, using Dukes and TNM-7 systems. Of the 68 patients, 3%, 9%, 86.5% and 1.5% presented at TNM stages I, II, III, and IV respectively Table 8 below.
Table 8: Pathological staging of 68 cases of colorectal cancer

<table>
<thead>
<tr>
<th>Dukes stage</th>
<th>Number</th>
<th>%</th>
<th>TNM Stage</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dukes A</td>
<td>7</td>
<td>10</td>
<td>Stage I</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Dukes B</td>
<td>39</td>
<td>57</td>
<td>Stage IIA</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td>Dukes C</td>
<td>16</td>
<td>24</td>
<td>Stage IIC</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Dukes D</td>
<td>6</td>
<td>9</td>
<td>Stage IIIA</td>
<td>39</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stage IIIB</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stage IIIC</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stage IVB</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>100</td>
<td></td>
<td>68</td>
<td>100</td>
</tr>
</tbody>
</table>

Designating TNM stage I and II along with Dukes A and B as early stages, TNM stage III and IV as well as Dukes C and D as advanced stage, following groups were found (Table 9).

Table 9: Comparison of staging using TNM and Dukes system

<table>
<thead>
<tr>
<th>Stage</th>
<th>TNM system</th>
<th>Dukes staging system</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
</tr>
<tr>
<td>Early stage</td>
<td>8</td>
<td>11.8</td>
<td>46</td>
</tr>
<tr>
<td>Advanced stage</td>
<td>60</td>
<td>88.2</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>100</td>
<td>68</td>
</tr>
</tbody>
</table>

Using the TNM staging, sixty (88.2%) patients presented with advanced disease whereas eight (11.8%) presented in early stage; on the other hand using the Dukes staging system, twenty two (32.3%) patients presented with advanced disease whereas forty six (67.7%) presented in early stage Table 9.
Majority of our patients could not be assigned a pathological stage due to the nature of the biopsy, for instance 134 (66.7%) patients underwent endoscopic biopsy for diagnostic purpose and no eventual resection of the cancer was performed because it was deemed inoperable. Patients on whom staging was applicable, TNM staging system has shown to classify patients in early stage 11.8% in comparison to Dukes staging system which classified 67.7% of them in early stage and this difference is strongly statistically significant (p=0.0001). Males presented in later stages than women 54.09% against 45.9% respectively but this difference was not statistically significant, p=0.844.

**Comorbidities associated with CRC**

HIV/AIDS was relatively common comorbidity but least associated with colorectal cancer 38.0% (Table 10). However the association with CRC was not statistically significant (P=0.688). The same applies with diabetes mellitus and hypertension whose P value was 0.424 and 0.266 respectively.

**Table 10: Distribution of comorbidities associated with colorectal cancer**

<table>
<thead>
<tr>
<th>Type of comorbidity</th>
<th>Frequency</th>
<th>Percent</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>27</td>
<td>38</td>
<td>0.688</td>
</tr>
<tr>
<td>Negative</td>
<td>44</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>21</td>
<td>29.7</td>
<td>0.266</td>
</tr>
<tr>
<td>Normal</td>
<td>50</td>
<td>70.3</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>23</td>
<td>32.3</td>
<td>0.424</td>
</tr>
<tr>
<td>Negative</td>
<td>48</td>
<td>67.7</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>71</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

In this study, colorectal cancer accounted for 5% of all malignancies diagnosed at Muhimbili national hospital. This concurs with the figure reported by the study done at CUHAS in Tanzania by Chalya et al, (37). In Libya it was the second most common malignant tumor accounting for 10% and 9% in males and females respectively (53). This two fold increase in proportional rate could be attributed to different geographical location and hence different diet and habits.

The mean age in this study was 50.2 years which corroborates that of 44.3, 49.7, 51 and 52.3 years reported in Nigeria, Kenya, Egypt and Iran respectively (53,54). The age incidence of CRC in Tanzanian population as well as other African countries, is lower compared to developed countries, about 10 years difference has been reported in many studies (56), this discrepancy could be attributed to well established complex interplay between non modifiable and modifiable risk factors peculiar to African population.

Peak age in our population was between 45 years to 59 years, this figure differs from that reported by Fatimah et al (57), in Nigeria, they found a peak of 60-69 age group which is relatively close to that of American population.

This difference may be due to different geographical location, racial variations, diet, alcohol consumption and may be Nigerian population has adopted Western lifestyle as well as better health systems accessibility compared to Tanzanian population which was made exclusively of black Africans.

There appears to be an increasing number of CRC occurring in the young in our study population since 27.8% were less than 40 years of age. Reports from other parts of Africa showed similar findings. A higher frequency of cancer in young age groups which also carries poor prognosis has been reported in the lower end of the oesophagus and cardia of the stomach (63, 64, 65). In Zimbabwe Katsidzira et al, (59) found 32% below 50 years with a remarkable ethnic dissimilality. CRC in younger age has been shown to present a diagnostic and therapeutic problem and prognosis tends to be less favorable.
In Uganda, Gondos et al. reported the 5-year survival rate of 8.3% for Ugandan population compared with 54.2% for black American patients (60). Near similar results found in Egypt by Gado where 25% of cancers occurred in patients aged less than 40 years.

Data from the West emphasized that less than 20% of CRCs occur less than 50 years but in other survey from Iran 34.5% of patients were below 50 years of age. CRC was detected in patients aged 40 years or younger in 2–6% of CRC cases in Italy, France and Taiwan and in 17–36% in Saudi Arabia, Sudan and Iran. All these data reflects that the colorectal cancer in Middle East and Africa is more common in the young compared to Western countries.

Agrawal recommended screening of African Americans at a younger age (45 rather than 50 years) as they were found to have a higher incidence of developing colorectal cancer at a younger age. Colorectal cancer affecting the younger population (<40 years old) is associated with poor prognosis (61). Dukes and Bussey suggested a much higher rate of lymphatic metastasis in patients less than 40 years of age due to a faster disease progression in young patients (53).

Miyake and Bedikian demonstrate that the 5 year survival rate for young patients (30 years old or younger) is only 25–30%. The need for early recognition of CRC in young adults is highlighted by the greater incidence of advanced disease and the high treatment failure rate. However, if detected in early stages, young patients with Dukes’ stage A or B lesions have better overall 5 year survival rates. On the other hand we demonstrated the highest incidence in the age group 52- 56 years (62.2%) which coincides with that reported by Ahmed et al. but differs from David et al. whose peak incidence was at 75 years due to the disparities of genetic and environmental factors in the study the population (61).

The above findings underscore the fact that young age is not only associated with poorer prognosis, but other factors like low index of suspicion of cancer in this age group by health practitioners, could also lead to late medical discovery.
Similar to other studies, our findings showed that males were more than females although male to female ratio was almost similar (1.1: 1) which can be compared to that shown by Verschueren et al, on the other hand Guraya showed a different male to female ratio of (4: 1). However the reasons for this were unclear (35, 36).

In terms of location of CRC, our study showed CRC to be located in order of frequency, in rectum, right hemicolon, and left hemicolon in 63.7%, 17.9% and 12.9% of cases respectively. This was in keeping with the report of Saidi et al, (55) in Kenya which showed a higher frequency in the rectum (79%). Differences in frequency of CRC in colonic subsites and rectum could be due to different transition times during embryogenesis, physiological functions and genetic mutations thus leading to different etiologic factors, furthermore because rectal tumors are readily accessible by digital rectal examination, they are more easily diagnosed which may lead to a higher detection frequency.

Tumor grade pattern in our study was mainly moderately differentiated adenocarcinoma (62.3%), followed by poorly differentiated (29.7%) then well differentiated adenocarcinoma (7%), in a similar study in Saudi Arabia moderately differentiated was the most common followed by poorly differentiated adenocarcinoma but in Sudan Mohamed et al.(23) showed well differentiated adenocarcinoma to be the commonest (45%) followed by poorly differentiated adenocarcinoma (26.7%), this differential pattern might be due to inherent different methodology and subjectivity in assigning grade of CRC otherwise grades hence biological behavior as well as location of the tumor were similar (36).

The determination of tumor grade has inherent subjectivity. Many studies have demonstrated that a 2-tiered grading system, which brings together well and moderately differentiated to low grade (50% gland formation) and defines poorly differentiated as high grade (<50% gland formation), reduces inter-observer variation and improves prognostic significance . Even though it is a controversial topic, tumor grade is generally considered as a stage-independent prognostic variable, and high grade or poorly differentiated histology is associated with poor patient survival. It should be emphasized, however, that histological grading should apply only to conventional adenocarcinoma. Some of the histologic variants, may show high grade
morphology but behave as low grade tumors because of their microsatellite instability (MSI) status (23, 36).

Our findings demonstrate 23% of CRC coexisting with villous adenomas emphasizing the sequential progression of the latter to give rise to CRC. Adenomas are by definition, clonal lesions that exhibit at least low grade dysplasia characterized by enlarged, hyperchromatic and elongated (pencillate) nuclei arranged in a stratified configuration along the basement membrane (Figure 5.2). The adenomatous cells may show mucin depletion and increased apoptotic activity. Conventional adenomas are subclassified as tubular, tubulovillous and villous based on their architectural features.

Tubular adenomas are made of simple crypt like dysplastic glands and contain <25% villous component. Villous adenomas express >75% villous component that resemble finger-like projections. Tubulovillous adenomas are considered as intermediate lesions with 25-75% villous component.

Adenomas that are large in size (>1 cm) or predominantly villous, or contain high grade dysplasia are considered “advanced adenomas”, which require more aggressive endoscopic surveillance (34).

This study has shown a predominance of poor prognosis tumors like mucinous adenocarcinoma (14.4%) and signet ring cell carcinoma (4.5%) and these poor prognostic entities were found to be associated with young age group (below 40 years), which was statistically significant, p=0.002. Moreover, signet ring carcinoma showed a slight female and right site predilection, reflecting its counterpart signet ring carcinoma of the stomach reported to occur more commonly in the pyloric region in comparison to other sites with female predilection (58).

Our findings are in keeping with what Mwakyoma et al, (8) found. They reported a prevalence of mucinous adenocarcinoma of 6.9% which was associated with young age.
Findings in this study are quite high highlighting a two-fold increase in the last two decades. This upsurge may be attributable to increased awareness in seeking medical care though national cancer control programs like screening are still elusive in this country.

Mucinous adenocarcinoma is a special type of colorectal carcinoma defined by >50% of the tumor volume composed of extracellular mucin. Tumors with a significant mucinous pools (>10%) but <50% are usually termed adenocarcinoma with mucinous features or mucinous differentiation (61).

Mucinous adenocarcinoma typically are characterized by large glandular structures with pools of extracellular mucin (Figure 5.3). A variable number of individual tumor cells, including signet ring cells, may be seen. The prognosis of mucinous adenocarcinoma compared to that of conventional adenocarcinoma, has been controversial among different studies. Many mucinous adenocarcinomas occur in patients with hereditary nonpolyposis colorectal cancer (HNPCC) and hence represent high level MSI (MSI-H) tumors (12). These tumors are regarded as to behave in a low grade fashion. In contrast, mucinous adenocarcinomas that are microsatellite stable (MSS) are expected to behave more aggressively, particularly when detected at an advanced stage (61).

Our youngest patient was a 12-year-old boy presented with complaints of severe pain in the abdomen left side, per rectal bleeding with constipation for the last six months without any predisposing factor. During this period he also gave a history of weight loss, he consulted general local physician at Mwananyamala but symptoms were not relieved.

So he was referred to MNH. There was no family history of polyposis, colon cancer and past medical history was also non-significant.

On examination, there was lumpish feel on left side of abdomen. Computed tomography of abdomen revealed enhancing mass at splenic flexure with marked narrowing of the lumen causing bowel obstruction without evidence of lymphadenopathy. After thorough clinical examination he underwent colonoscopy and stenosing growth was observed in sigmoid and
descending colon portion. Biopsy was taken from the growth and histopathological examination suggested diagnosis of mucinous adenocarcinoma. Thus surgical resection was planned. After complete diagnostic work up and preoperative evaluation, left hemicolecetomy with end-to-end anastomosis with complete nodal clearance was done. Tumor was resected with portion of colon about 20 cm long. On gross examination of specimen, growth was exophytic with glistening surface measuring 4x2x1 cm showing protrusion within lumina obliterating it partially.

The lesion involved full thickness of intestinal wall. On cut surface, lesion was greyish and mucinous on appearance. Microscopic examination revealed extensive areas of mucin secretions with few narrow glands lined by cuboidal epithelium showing hyper chromatic and pleomorphic nuclei. The tumor was infiltrating through muscularis propria reaching beyond serosa into adipose tissue. Signet ring cells were also present. Regional lymph nodes were involved (Figure 5.3). All these findings confirmed the diagnosis of mucinous adenocarcinoma of colon. The tumour was classified as Duke’s C stage. He was referred to Ocean road cancer institute for adjuvant chemotherapy.

Noh et al, (63) reported 15-year old colon cancer patient with a 10-year history of ulcerative colitis. This highlights screening with colonoscopy for children with long-term history of predisposing factor like Inflammatory Bowel Disease. Other studies documented that 10% of the pediatric patients have predisposing factors for CRC. Genetic factors increasing risk of CRC are familial polyposis of colon, Gardner’s and Bloom’s syndrome, Turcot’s and Peutz - Jegher’s syndrome. But in our case, this boy had no significant risk factor such as positive family history of any type of malignancy or polyposis in his first and second-degree relatives, no past history of IBD and no specific dietary factor.

Signet ring cell adenocarcinoma, in contrast to that in the stomach, is not uncommon in the colorectum, representing 4.5% of all CRC. Similar to mucinous carcinoma, signet ring cell carcinoma is defined by the presence of >50% of tumor cells showing signet ring cell features characterized by a prominent intracytoplasmic mucin vacuole that pushes the nucleus to the periphery (Figure 5.4). Signet ring cells may show an infiltrative growth pattern or are present
within the pools of extracellular mucin. Signet ring cell carcinoma is by definition, a poorly differentiated (high grade) and carries a worse outcome than conventional adenocarcinoma. However, some signet ring cell carcinomas may be MSI-H tumors and thus may behave as low grade tumors biologically (40, 58).

Medullary carcinoma (MC) is relatively rare in our study population, constituting 0.5% of all CRC. This tumor is characterized by sheets of epithelioid neoplastic cells with large vesicular nuclei, conspicuous nucleoli, and abundant cytoplasm. It typically has a pushing border on resection specimens (Figure 5.14), and is characteristically associated with marked tumor-infiltrating lymphocytes (Figure 5.15). Our study shows that medullary carcinoma is probably much less common than suggested by Lanza et al. or other studies, mainly due to the lack of uniform criteria for defining this histological type in the other studies. For example, Lanza et al, used the criteria of >70% solid component and lack of nuclear pleomorphism as the criteria for MC, with tumor samples showing varying amounts of glandular differentiation. Our study used the ICD-O-3 coding mentioned in the WHO classification, which identifies medullary carcinomas as tumors with sheets of malignant cells with abundant pink cytoplasm, prominent nucleoli, vesicular nuclei and intraepithelial lymphocytic infiltrate without any specific mention of the percentage of the differentiated or solid component (29).

Medullary carcinoma is a distinctive histologic subtype that is strongly associated with MSI-H. It usually has a favorable outlook despite its poorly differentiated or undifferentiated histology. The favorable prognosis of MCs in general is probably because of the fact that they still do retain some intestinal differentiation and coexisting lymphoid infiltration, indicating host reaction against tumor. However, such a conclusion would require further studies in the future including immunohistochemical analysis with regard to the specific subsets of MCs (29, 40).
The colon and rectum are uncommon sites of involvement of Non-Hodgkin’s lymphoma. In our study population we noted a 57 year-old, male patient, HIV positive who presented with a history of 6 month abdominal mass, pain and weight loss, colonoscopy revealed an edematous caecal mucosa causing external compression, biopsy was not conclusive, exploratory laparotomy was done and resection specimen was submitted for histology evaluation, grossly a distinct caecal tumor was found and microscopically the cells were monotonous with irregular nuclear membranes and prominent nucleoli with easily found mitotic activity(Figure 5.6), histochemical and immunohistochemistry staining were performed and revealed the tumor to be PAS negative (Figure 5.8), reticulin consistent with lymphoid stroma (Figure 5.9), CD45 positive, CD20 positive (Figure 5.10), pan cytokeratin negative, diffuse large B cell lymphoma was reported as a final diagnosis. Similarly to other studies, the most frequently involved colonic site at diagnosis is the ileocaecal region, followed by the caecum, the sigmoid and the rectum, such site predilection is due to rich lymphoid tissue. The etiology of diffuse large B cell lymphoma is unknown, but some risk factors and predisposing conditions have been identified such as immunodeficiency like the one in our study population and inflammatory bowel diseases.

Despite the severe luminal narrowing, lymphoma is less likely to cause obstruction because it does not elicit a desmoplastic response and submucosal lymphoid infiltration weakens the muscularis propria of the wall.

The diagnosis of primary colonic lymphomas (PCL) was initially established in 1961 and included the following diagnostic five criteria:

No palpable superficial lymphadenopathy; no enlargement of the mediastinal lymph nodes on chest X-ray; normal white blood cell count; predominance of the bowel lesion, and adjacent lymph nodes affected at laparotomy; absence of any tumor in the liver or spleen at laparotomy. Our patient’s disease met all these criteria preoperatively (40).
The most common histological subtype of colorectal lymphoma is diffuse large B-cell lymphoma, as our patient disease. Other histologies include follicular lymphoma, Burkitt lymphoma and Mantle cell lymphoma.

The majority of CRC in this study presented in TNM stages II (9%) and III (86.5%) or Dukes B (57%) and C (24%). Thus 97% of patients with CRC presenting at MNH were in advanced stages which limits surgical interventions. This figure is in keeping with the finding (96.7%) of Phillipo et. Al (37) at CUHAS in Tanzania, only 3% of patients were diagnosed in early stages where curative intent was guaranteed. This finding is in marked contrast to the annual incidence rate of European countries of 8.6% (15). The reasonable explanation for this difference is ascribed to the Westernized lifestyle and lack of fully established and functioning screening programs in many African countries.

TNM staging system is known to classify CRC in prognostically different strata, in this study TNM-7 proved to be superior to older Dukes system in terms of stratifying patients in reproducible and clinically different classes, this has also been reported by other authors (39, 46, 47).

Immunosuppression by HIV/AIDS was the leading comorbidity in our population; however the association with CRC was not statistically significant, moreover, the degree of association is unreliable because information about sero-status of most of our cases was not available. As reported by other authors (62), HIV positive patients could be at higher risk of developing CRC in our population, which is, younger age, a special group to be considered in case of any eventual health program on colorectal cancer. Berretta et al. in Italy, showed that HIV-positive CRC patients compared to HIV-negative patients have a poorer performance status, an unfavorable Dukes’ stage, higher grading and shorter survival. Morever Yamaji et al, in Japan in multivariate analysis, they showed female sex, increased BMI, hypertension, and diabetes, were more associated with colorectal cancer. Smoking, aspirin use, and coronary artery disease, on the other hand showed an inverse relationship (19, 20).
Comorbidities like HIV/AIDS, diabetes and hypertension were common in our study population, but they didn’t achieve a significance level to explain a causal relationship with CRC may be due to incomplete data on these variables, but we still hypothesize that their constellation and synchronous or metachronous occurrence with CRC are responsible advanced stages, cancer types of grim prognosis and poor outcome.

A further prospective study is needed to draw a reliable eventual causal relationship of these comorbidities with CRC.
CONCLUSIONS AND RECOMMENDATIONS

Conclusions
CRC is changing trend in Tanzania. The age, sex and morphological characteristics are similar to that in other reports from Africa, but different from Western countries. Morphologic types of poor prognosis are not uncommon in young age, with advanced stages at presentation. However for the best of our knowledge, little is known about eventual specific etiopathologic mechanism that could account for this presentation. Molecular studies are paramount to shed light on molecular subtypes and prognostic features associated with CRC in our young population. A significant number of the Tanzanian population with CRC have precursor adenomatous lesion which can be discovered and treated earlier before giving rise to cancer.

Recommendations
To the Ministry of Health:
- To establish a functional cancer registry which can be helpful to determine a nationwide magnitude of colorectal cancer.
- To prioritize screening programs of colorectal cancer starting at younger age compared to western countries.
- To Muhimbili University of Health and Allied Sciences:
  - To support advanced researches (genetic and molecular) on colorectal cancer in younger age group, this could be helpful in personalized therapy.
  - To conduct a prospective study this can establish a reliable relationship between HIV status and CRC in young age group.

To clinicians at MNH and other Hospitals:
- To increase index of suspicion of colorectal cancer in young age patients presenting with vague abdominal pain and diarrhea.
To investigate systematically HIV status to every patient with gastrointestinal symptoms.

To pathologists at MNH:
- To adopt TNM staging together with Dukes staging system, to deliver a more predictive and prognostic clinical information on resection specimens of colorectal cancer.
REFERENCES


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### Appendix I: Data Collection Sheet

#### I. Patient’s Particulars

1. Data Collection Sheet Number:  
2. Hospital Registration Number:  
3. Age at Diagnosis (Years):  
4. Race: □ African  □ Asian  □ Caucasian  □ Mixed  
5. Sex:  
   □ M  □ F  
6. Date of Diagnosis (dd/mm/yyyy):  
7. Region of Residence:  

#### II. Clinical data

1. Type of the biopsy:  
   a) Endoscopic  
   b) Excision  
2. Site of the biopsy:  
   a) right colon  
   b) left colon  
   c) rectosigmoid colon  
   d) rectum
3. Comorbidities
   a) Diabetes  
   b) Hypertension  
   c) HIV/AIDS  
   d) Non comorbidity

III. Histological findings

1. Type of the colorectal cancer (mention)..............................................

2. Dukes’s stage   A  B  C  D

3. Tumour grade   I   II   III   IV

4. TNM stage (mention)...........
Appendix II: Standard Operating Procedure (SOP) for H&E histochemistry

1. Deparaffinize sections, hydrate through graded alcohol to water

2. Remove fixation pigments if necessary

3. Stain in alum hematoxylin for a suitable time.

4. Wash well in running tap water until section ‘blue’ for 5 minutes

5. Differentiate in 1% acid alcohol (1% HCL in 70% alcohol) for 5-10 seconds.

6. Wash well in tap water until sections are again ‘blue’ (10-15 minutes)

7. Stain in 1% eosin Y for 10 minutes.

8. Wash in running water for 1-5 minutes

9. Dehydrate through alcohols (70, 95, 100%), clear and mount
Appendix III: SOP for reticulin histochemistry

1. Deparaffinize and hydrate to distilled water.

2. Potassium permanganate solution, 5 minutes

3. Wash in water

4. 5% oxalic acid until clear

5. Wash in distilled water

6. Iron alum solution, 10 minutes

7. Wash in running tap water, rinse in distilled, 3 changes

8. Silver solution, 7 dips, shake excess solution off slides

9. Distilled water, 2 changes, 3 quick dips each

10. 10% formaldehyde solution until gray black, 30 seconds

11. Wash in distilled water

12. 0.5% Gold chloride, 1 minute

13. Rinse in distilled water

14. 5% hypo, 1 minute.

15. Wash in tap water

16. Nuclear-fast red solution, 5 minutes

17. Wash in running tap water

18. Dehydrate, clear, and coverslip.
Appendix IV: SOP for PAS histochemistry

1. Deparaffinize and hydrate to distilled water

2. Place slides into 0.5% Periodic acid for 5 minutes

3. Rinse in distilled water

4. *Schiff’s Reagent, microwave HIGH power, for 45 - 60 seconds, until deep magenta

5. Wash in running tap water for 5 minutes

6. Counterstain in hematoxylin for 3 minutes

7. Wash in tap water, blue hematoxylin, rinse in distilled water

Appendix V: SOP for Immunoperoxidase stains

Make tissue block sections 4 μm thick, put on immunohistochemistry (IHC) slides

1. De-wax by placing the slides on hot plate at 60°C for 50 minutes
2. Immerse the slides in xylene in 2 batches for 5 minutes each.
3. Rehydrate through alcohols (100, 95, 70%).
4. Rinse the slides with deionized water.
5. Surround the tissue with a hydrophobic barrier using a barrier pen.
6. To quench endogenous peroxidase activity, incubate the sample with 2 drops of peroxidase blocking reagent (3% H₂O₂ in water or methanol) for 15 minutes.
7. Bring the slides into water for 5 minutes.
8. Prepare antigen retrieval solution to unmask the antigens
9. Heat antigen retrieval solution in pressure cooker (not closed) until it starts boiling
10. Place slides in antigen retrieval solution in pressure cooker and close the lid
11. Time for 2 minutes at full pressure
12. Remove the slides from pressure cooker and place in water (never allow slides to dry)
13. Add Tris buffer solution (TBS) wash buffer for 3 minutes
14. Drain slides of TBS, and wipe away any excess wash buffer, add the primary antibody
15. Incubate the section with the antibody for 30 minutes
16. Wash 3 times with wash buffer for 5 minutes each, and drain the slides.
17. Incubate the sample with 1-3 drops of HRP (Horseradish peroxidase) for 30 minutes.
18. Rinse with wash buffer 3 times for 15 minutes each and drain the slides.
19. Incubate the sample with 1-3 drops of biotinylated secondary antibodies (HRP) for 30-60 minutes (time depends on thickness of the section).
20. Rinse and wash 3 times in wash buffer (TBS) for 2 minutes each.
21. Calculate the required working volume of DAB Chromogen Solution given that 100-200 µL is required to cover the entire tissue section on a single slide. Make up DAB: 1 drop of DAB for each 1 mL of substrate buffer.

22. Add 1-5 drops of DAB Chromogen Solution to cover the entire tissue section and incubate for 3-20 minutes. Colored precipitate will localize to the sites of antigen expression as the chromogenic substrate is converted by HRP enzyme into insoluble end product.

Note: DAB and AEC are hazardous materials. Gloves and safety glasses should be worn and all steps performed inside a fume hood.

23. Rinse the sample with wash buffer (TBS) 3 times for 10 minutes each.
24. Rinse in deionized H$_2$O and drain the slides.
25. Counterstain in hematoxylin for 10 seconds.
26. Quick dip in 1% acid alcohol, 2 dips
27. Blue in warm water for 2 minutes
28. Dehydration through ascending concentrations of alcohol (70, 95, 100%)
29. Clear in xylene in 2 batches for 3 minutes each
30. Coverslip
31. Visualize staining of tissue under a microscope using a bright-field illumination.