Merowe University of Technology

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The prevalence of characterization of the Human epidermal growth factor receptor 2 expiration among the Colorectal Cancer patients, in Sudan (2025)

A thesis submitted for the fulfillment of Ph.D. Degree in Medical Laboratory Sciences in (Histopathology and Cytology)

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1. Introduction:

1.1. Background:

Colorectal cancer (CRC) is one of the most common and lethal malignancies worldwide. Its development and progression are influenced by a complex interplay of genetic, epigenetic, and

environmental factors. Recent advancements in molecular biology have provided a deeper understanding of the mechanisms underlying CRC and have paved the way for novel diagnostic, prognostic, and therapeutic approaches. These molecular strategies have become pivotal in improving patient outcomes [1].

The backbone of treatment for ~ 95% of mCRC, characterized by proficient mismatch repair/microsatellite stability (pMMR/MSS), thus excluded from treatment with immune checkpoint inhibitors, still relies on cytotoxic drugs such as 5-fluorouracil, oxaliplatin, and irinotecan combined with a biological agent, being an anti-EGFR or anti-VEGF agent according to clinical–molecular features. However, their efficacy is limited over time, making it important to plan a continuum of care integrating multiple therapeutic options as the cornerstone of mCRC management. In this context, novel targeted therapies aimed at extending treatment and improving survival are needed. Among the most recent targeted therapies endorsed by the European Society for Medical Oncology (ESMO) and the National Comprehensive Cancer Network (NCCN), combinations targeting human epidermal growth factor receptor 2 (HER2) have emerged as a key option for patients with amplification of the corresponding gene (also known as ERBB2) [2, 3].

CRC typically arises through the stepwise accumulation of genetic alterations. The adenoma-carcinoma sequence, which accounts for the majority of CRC cases, involves mutations in key oncogenes and tumor suppressor genes. The earliest event is often a mutation in the APC gene, leading to aberrant activation of the Wnt/β-catenin signaling pathway. This is followed by activating mutations in the KRAS gene, which promote uncontrolled cell proliferation. Inactivation of the tumor suppressor TP53 and mutations in genes involved in TGF-β signaling, such as SMAD4, occur at later stages of tumor progression [4]. Another molecular pathway implicated in CRC is the microsatellite instability (MSI) pathway, characterized by defective DNA mismatch repair (MMR) systems. This pathway accounts for approximately 15% of CRC cases and is associated with hypermutability, leading to a distinct molecular and clinical phenotype. Lynch syndrome, a hereditary form of CRC, is strongly linked to MSI [5]. Additionally, the CpG island methylator phenotype (CIMP) represents an epigenetic mechanism contributing to CRC. Hypermethylation of promoter regions in genes such as MLH1 silences critical tumor suppressors, further driving carcinogenesis [4].

HER2 is located on chromosome 17q21 and encodes a 185 kD transmembrane glycoprotein receptor that is activated by the phosphorylation of tyrosine residues. HER2 activation initiates

various signal cascades such as the MAPK, PI3K/AKT, and PKC pathways, which are essential for cell differentiation, proliferation, and survival during fetal development [6-8]. In neoplastic cells, dysregulation of the mentioned pathways and also increased expression of HER2 promotes tumor cell growth, progression, and migration [9]. Overexpression of the HER2 receptor is detected in 25% - 35% of breast cancer patients and has been known as an important prognostic and predictive factor [10].

HER2 overexpression/amplification accounts for 3% (unselected) to 5% (RAS and BRAF wild-type molecular profiles) of mCRCs and occurs predominantly in left-sided colon and rectal adenocarcinomas, being associated with specific sites of metastases such as the central nervous system [11,12,13]. HER2 gene amplification leads to aberrant signaling of downstream pathways, including the mitogen-activated protein kinase (MAPK)-dependent, the phosphoinositide 3-kinase (PI3K)-dependent, and the Janus kinase/signal transducer and activator of transcription (JAK/STAT)-dependent pathways, ultimately promoting tumor proliferation and survival [14].

The prognostic impact of HER2 amplification has been evaluated with controversial results [15,16].

However, in a recent large individual patient data pooled analysis of eight randomized trials in untreated patients with MSS, RAS/BRAF WT mCRC, HER2 amplification/overexpression (by means of immunohistochemistry ± in situ hybridization and/or NGS on tumor or circulating DNA) was found as an independent negative prognostic factor [17].

Ross et al (2001) reviewed the HER2/neu in gastrointestinal tumors. They stated that either HER-2/neu protein overexpression or gene amplification is associated with approximately one-fourth of all gastrointestinal tract malignancies [18].

Both the ESMO Living and the NCCN guidelines recommend HER2 testing in mCRC according to the HERACLES criteria or through next-generation sequencing (NGS) in cases of suspected or confirmed distant metastases [2,3]. However, unlike in breast and gastric cancer, HER2 positivity lacks a standardized definition, as key clinical trials have adopted different classification criteria [19].

In 2015, validated HER2 overexpression criteria in mCRC for enrolling patients into the phase II HERACLES trial were proposed. According to these criteria, HER2 positivity is defined by

intense circumferential, basolateral, or lateral immunohistochemical staining (IHC 3+) in ≥ 50% of tumor cells. If the staining is observed in > 10% but < 50% of cells, a positive in situ hybridization (ISH) result (*HER2/CEP17* ratio ≥ 2 in ≥ 50% of cells) is required to confirm HER2 positivity. Similarly, moderate circumferential, basolateral, or lateral staining (IHC 2+) in ≥ 50% of cells also requires a positive ISH result to be considered HER2 positive. Compared with breast and gastric cancer, the HERACLES classification demands a higher proportion of tumor cells to exhibit staining (≥ 50% versus ≥ 10%), and it allows a broader range of staining patterns. However, the ISH positivity criteria are similar across these tumor types. Nowadays, the HERACLES criteria are widely used as diagnostic criteria across prospective clinical trials of anti-HER2-based treatments [20].

A key challenge in HER2 detection in mCRC is intratumoral heterogeneity, which can be genetic (a mix of HER2+ and HER2− cells) or nongenetic (HER2-amplified cells that do not express HER2 protein). Notably, HER2 intratumoral heterogeneity is relatively common in mCRC: a gene-protein assay demonstrated that 22% of HER2-positive samples displayed heterogeneity, with rates varying from 16% in IHC HER2 3+ to 60% in IHC HER2 2+ [21].

Interestingly, in the MOUNTAINEER trial, central HER2 testing was performed using IHC/ISH and/or tissue- or blood-based NGS. The concordance rates were 92.6% between IHC/FISH and tissue-based NGS, 81% between blood- and tissue-based NGS, and 79.5% between IHC/FISH and blood-based NGS [22]. Taken together, these findings suggest that multiple methodologies can be employed for HER2 assessment in mCRC. Notably, gene-based approaches may offer advantages by overcoming intratumoral heterogeneity with ctDNA also potentially overcoming heterogeneity between primary and metastasis and by increasing detection rates. As a result, incorporating NGS and liquid biopsy into diagnostic workflows could further refine patient selection for HER2-targeted therapies [23].

In Africa, CRC is still the fifth most common malignancy, with a higher incidence in males than females, and the highest risk is found in North, Southern, and East Africa [24]. Libya reports the highest age-standardized rates and age-specific incidence (ASRs) for CRC among the North African countries, and it accounts for the first malignancy in Libyan men [25,26]. In Sudan, a retrospective study was conducted in Ibn Sina Hospital (Sudan). Seventy-three (73) patients of colorectal cancer who presented in the period from January 2010 to December 2012 were found that more than 17 % of the study population was below the age of 40 years, and 43.84% was below 50 years. The male-to-female ratio was 1:1.02. Rectal bleeding is the

commonest presenting symptom, and well-differentiated adenocarcinoma is the dominating tumor grade. About 8.3 % of patients presented with liver metastasis [27].

Despite the clear prognostic impact and therapeutic potential of HER2 status in colorectal cancer being validated in multiple international trials [17, 12], this actionable molecular marker remains largely uncharacterized within the Sudanese population. Colorectal cancer in Sudan presents unique epidemiological features, often affecting younger patients [27], yet local data on specific molecular pathways, including HER2 overexpression and amplification, are critically sparse. This data deficit represents a significant barrier to implementing modern precision oncology, preventing the systematic identification of patients eligible for HER2-targeted therapies endorsed by leading bodies like the ESMO and NCCN [2, 3]. While international trials and guidelines (ESMO, NCCN) validate HER2 as an actionable biomarker, there are almost no systematic data from Sudan a gap that seriously limits local access to targeted therapies. Therefore, this study is rigorously designed to address this knowledge gap by conducting a comprehensive molecular and immunohistochemical assessment of HER2 in a cohort of Sudanese CRC patients. By establishing the prevalence and clinicopathological correlation of HER2 status using validated scoring systems [20], this research will provide the foundational local evidence necessary to elevate diagnostic standards, guide therapeutic decision-making, and successfully integrate life-extending targeted treatments into the continuum of care for colorectal cancer in Sudan.

1.2. Problem Statement:

Colorectal Cancer (CRC) remains a highly prevalent and lethal malignancy in Sudan and across Africa, often presenting in younger patient demographics [24, 27]. While global oncological guidelines, particularly for metastatic CRC (mCRC), increasingly incorporate HER2-targeted therapies as a crucial treatment option for the 3-5% of *RAS* and *BRAF* wild-type patients who exhibit HER2 amplification [2, 3, 11], there is a significant data deficit in the local context. The molecular landscape of CRC in Sudanese patients including the prevalence, characteristics, and clinical relevance of HER2 status remains largely undetermined, building upon limited retrospective findings in the region [27]. This critical lack of local epidemiological and molecular data means that eligible patients in Sudan who could benefit from these specific, life-extending precision medicine treatments are currently **not being systematically identified or offered optimal therapeutic pathways**, resulting in a critical gap between international standards of care and local practice.

1.3. Justification

This study is critically important for elevating the standard of care for CRC patients in Sudan by enabling the adoption of precision oncology. HER2-targeted combinations offer a validated, survival-extending option for patients who typically have limited therapeutic alternatives after failing standard cytotoxic and anti-EGFR treatments. By comprehensively establishing the local prevalence and clinical profile of HER2 positivity through standardized IHC and molecular techniques, the research will provide the foundational evidence required for local oncologists to mandate routine HER2 testing as part of the diagnostic workflow. Furthermore, the findings will inform local healthcare policy and infrastructure planning, directly facilitating the integration of international anti-HER2 treatment protocols and ensuring that Sudanese CRC patients have equitable access to globally recognized, targeted therapeutic regimens, addressing a critical need identified in the region.

1.4. Research Questions:

1. What is the prevalence of HER2 expression among patients diagnosed with colorectal cancer in Sudan?
2. What are the patterns of HER2 expression (negative, equivocal, positive) in colorectal cancer tissue samples?
3. Is there an association between HER2 expression and clinicopathological characteristics of colorectal cancer patients (age, sex, tumor site, grade, and stage)?
4. Does HER2 expression vary according to histopathological features of colorectal cancer in the study population?

1.5. Objectives:

1.5.1. General Objectives:

To determine the prevalence and characterize the expression of Human Epidermal Growth Factor Receptor 2 (HER2) among colorectal cancer patients in Sudan.

1.5.2. Specific Objectives:

1. To determine the prevalence of HER2 expression among colorectal cancer patients.
2. To classify HER2 expression patterns in colorectal cancer tissue samples using immunohistochemistry.
3. To describe the sociodemographic characteristics of colorectal cancer patients included in the study.
4. To assess the association between HER2 expression and clinicopathological variables, including tumor site, histological type, tumor grade, and stage.
5. To evaluate the relationship between HER2 expression and histopathological features of colorectal cancer.

2. Materials and Methods

2.1 Study Design, Patient Selection, and Tissue Specimens

This study will be conducted as an analytical cross-sectional study. Archived formalin-fixed paraffin-embedded (FFPE) colorectal cancer tissue samples and corresponding patient clinical data will be analyzed to determine the prevalence and patterns of HER2 expression and to assess its association with clinicopathological characteristics. In patients who underwent surgical resection at a major clinical center in Sudan, and treated at **Merowe Medical City**. The study will include archived colorectal cancer cases diagnosed between 2024 and 2026 to ensure an adequate sample size. As an analytical cross-sectional study, all variables will be assessed at a single point in time per case.

Sample Size Estimation: The required sample size will be estimated using the formula

$N = p\,(1-p)Z^2/d^2$,

p = 0.05: The 5% prevalence estimate was selected based on reported HER2 amplification rates in contemporary mCRC cohorts Sartore-Bianchi et al., 2019 [13]. This prevalence is also consistent with recent trial screening rates used in HER2-targeted mCRC studies and with the range cited in the literature (3–5%). Where possible, we will perform subgroup analyses (e.g., RAS/BRAF wild-type) and report exact confidence intervals. If available sample numbers exceed 90, all additional eligible cases will be included to increase analytical power.

Where Z = 1.96 (for 95% confidence), p = 0.05 (expected prevalence of HER2 amplification in unselected mCRC based on published series; Sartore-Bianchi et al. 2019 [13] , and d = 0.05 (desired margin of error). Substituting:

$$N = \frac{(1.96)^2 \times 0.05 \times 0.95}{0.05^2} \approx 73$$

Inclusion Criteria:

- Confirmed histopathological diagnosis of invasive colorectal adenocarcinoma (according to WHO classification).
- Availability of adequate FFPE tissue blocks suitable for serial sectioning and subsequent Immunohistochemistry (IHC) and Molecular Profiling.
- Availability of comprehensive clinico-pathological data.

Exclusion Criteria:

- Non-epithelial colorectal lesions.
- Patients with prior malignancies or co-existing tumors.
- Patients who received pre-operative neoadjuvant chemotherapy or radiation therapy for CRC, as these treatments can alter HER2 expression status.

Data Collection:

Data will be collected using a **structured checklist** designed specifically for this study. Information will be obtained from histopathology laboratory records, patient medical files, and immunohistochemistry results (Appendix 2). Detailed clinico-pathological parameters will be retrospectively collected from medical records, pathology reports, and operative notes. These variables include gender, age at diagnosis, tumor location (left- vs. right-sided), tumor size, histological grade, presence of distant metastasis (pM stage), surgical resection margin status, vascular invasion, and perineural invasion.

2.2 Tissue Processing and Histopathological Review

Serial sections (4-micron thickness) will be prepared from the selected FFPE blocks. One section from each block will be stained with **Haematoxylin and Eosin (H&E)** for histopathological re-evaluation by two independent expert pathologists. Tumor grading will be performed according to the latest **WHO classification criteria**, and staging will be assessed using the **UICC guidelines (8th edition preferred)**. This step ensures the accuracy and consistency of the baseline diagnosis and grading.

2.3 Immunohistochemical (IHC) Staining for HER2

IHC staining will be performed at the **Merowe Medical City Histopathology Laboratory** using the **Zytomed™ kit (Zytomed)**, according to the manufacturer's instructions.

Methodology:

1. **Deparaffinization and Rehydration:** Tissue sections will be deparaffinized in xylene and rehydrated through a graded series of ethanol solutions.
2. **Epitope Retrieval:** Heat-induced epitope retrieval will be performed using **citrate buffer (pH 6.0)**.
3. **Peroxidase Blocking:** Endogenous peroxidase activity will be blocked with **3% hydrogen peroxide** to eliminate non-specific background staining.
4. **Primary Antibody Incubation:** Slides will be incubated with the primary **anti-HER2 antibody**.
5. **Visualization:** Application of a visualization reagent (secondary antibody and horseradish peroxidase-labeled polymer).
6. **Chromogen Development:** Color development will be achieved using **diaminobenzidine (DAB) chromogen**.
7. **Counterstaining:** Nuclei will be counterstained with **hematoxylin**.
8. **Mounting:** Sections will be dehydrated and mounted.

Controls: Positive and negative controls will be included in each batch of IHC staining. The positive control will be a known HER2-positive breast cancer case, and the negative control will be the same tissue with the primary antibody omitted (using only buffer).

2.4 HER2 Scoring System (IHC and Positivity Criteria)

IHC slides will be evaluated independently by two expert pathologists blinded to the clinical outcomes. The HER2/neu immunostaining will be scored based on the modified **HERACLES criteria** specifically validated for CRC, which differ from breast cancer criteria [21]. Cytoplasmic or background staining will be ignored, and only membranous staining will be graded:

Score	Staining pattern	Interpretation
0	No staining or incomplete membrane staining in < **50%** of tumor cells	Negative
1+	Faint/barely perceptible incomplete membrane staining in ≥ **10%** of tumor cells	Negative
2+	Weak to moderate complete or basolateral membrane staining in ≥ **50%** of tumor cells	Equivocal — requires ISH confirmation
3+	Intense complete or basolateral membrane staining in ≥ **50%** of tumor cells	Positive

Definition of HER2 Positivity: A case will be classified as **HER2 positive** if it meets one of the following criteria:

1. IHC score of 3+.
2. IHC score of 2+ **AND** a positive gene amplification status as confirmed by In Situ Hybridization (ISH) or Next-Generation Sequencing (NGS) (Molecular Profiling).

2.5 Molecular Profiling (ISH and PCR-Based HER2 Analysis)

Following immunohistochemistry (IHC) screening, all cases determined to be **IHC 2+ (equivocal)** will undergo confirmatory molecular testing to assess HER2 gene amplification. Molecular analysis will be performed at **Shandi Laboratory**, which is equipped to conduct PCR-based assays as well as ISH techniques.

2.5.1 In Situ Hybridization (FISH / CISH)

All **IHC 2+** cases will undergo **Fluorescence In Situ Hybridization (FISH)** or **Chromogenic In Situ Hybridization (CISH)** for assessment of HER2 gene amplification and calculation of the **HER2/CEP17 ratio**.

ISH Positivity Criteria:
A case will be considered **HER2-amplified** if:

- **HER2/CEP17 ratio ≥ 2.0**,
- In ≥ **50%** of tumor cells.

Equivocal or borderline ISH findings will be reviewed independently by two molecular pathologists. When required, repeat testing will be performed for confirmation.

2.5.2 PCR-Based HER2 Gene Amplification Analysis

Given the limited availability of NGS and the feasibility constraints in Sudan, **PCR will be the primary method used for molecular HER2 analysis.** Quantitative Polymerase Chain Reaction (**qPCR**) will be used to quantify **ERBB2 (HER2)** gene copy number.

a. Target Gene

The PCR analysis will focus specifically on the **ERBB2 (HER2)** gene.

b. Primers for HER2 (ERBB2)

Primers will be designed to amplify regions commonly evaluated for copy number gain in HER2:

- **HER2 Forward Primer:** 5'-AGGACCTGATGATGACGT-3'
- **HER2 Reverse Primer:** 5'-TCCAGGACTGGTGAGGTT-3'

Primer specificity will be confirmed using NCBI Primer-BLAST and validated against reference controls at Shandi Lab.

c. PCR Workflow and Conditions

PCR reactions will be conducted in **20 µL** volumes using SYBR Green or TaqMan master mixes according to reagent availability.

Cycling conditions:

- Initial denaturation: **95°C for 10 minutes**
- 40 cycles of:
    - Denaturation: **95°C for 15 seconds**
    - Annealing/Extension: **60°C for 60 seconds**

The **ΔΔCt method** will be applied to quantify relative HER2 gene amplification, using a reference housekeeping gene such as **RNaseP** or **β-actin**.

d. Quality Control Measures

Each PCR run will include:

- **Positive control** DNA from known HER2-amplified samples
- **Negative no-template control (NTC)**
- **Internal control gene** to normalize amplification
- **Technical replicates (duplicates/triplicates)**
- **Melting curve analysis** (for SYBR Green assays) to confirm specificity

PCR-based HER2 copy number results will be interpreted in conjunction with IHC findings and ISH results where available, acknowledging the methodological differences between copy number quantification and in situ amplification assessment.

2.6 Statistical Analysis

2.7 Ethical Considerations

This research will be conducted in strict accordance with the ethical principles outlined in the Declaration of Helsinki (as revised in 2013) and the International Ethical Guidelines for Biomedical Research Involving Human Subjects.

Ethical Approval: Formal agreement to conduct this research will be sought from the Research Ethics Board (REB) at Merowe University of Technology and from the respective Research Ethics Board (REB) and Northern state ministry of health of all included histopathology laboratories and hospitals in Sudan that provide archived tissue specimens. Research procedures will commence only after all necessary ethical clearances are secured.

Patient Confidentiality and Data Security: Patient confidentiality will be maintained throughout the study. All collected clinical and pathological data will be anonymized and coded immediately upon collection to protect patient identity. The key linking codes to patient information will be secured and accessible only to the principal investigator. FFPE tissue blocks will be handled and stored in compliance with local regulatory guidelines, ensuring patient privacy is protected at all stages. As a retrospective study utilizing archived tissues, the specific requirements for individual patient consent will be reviewed and justified based on the

ethical guidelines established by the local RECs, with a focus on waiver of consent for de-identified data.

3. Appendices:

3.1 Timeline and Implementation Plan

Task	Nov 2025	Dec 2025	Jan–Jun 2026	Jul–Dec 2026	Jan–Jun 2027	Jul–Sep 2027	Oct–Dec 2027
Proposal preparation & supervisor feedback							
Ethical approvals & institutional registration							
Case identification & retrieval of FFPE blocks							
Histopathologic review & H&E re-cutting							
IHC staining (HER2)							
ISH (FISH/CISH) for IHC 2+ (and QA on subset of 3+)							
DNA extraction & NGS (selected cases)							
Data cleaning & statistical analysis							
Thesis writing, submission & dissemination							

3.2 Budget and Resources

The following table provides a detailed breakdown of the estimated costs required for the successful completion of the research project, spanning the data collection and laboratory phases (2025–2026). All figures are provided in United States Dollars (USD).

Item	Details	Estimated Cost (USD)
Data Collection	Access fees, retrieval of archived FFPE tissue blocks, and secure transfer of clinical patient data from participating hospitals.	1,000
Transportation & Logistics	Travel and logistics expenses for the researcher and safe transport of tissue samples between the primary hospital, the Merowe Medical City Histopathology Lab, and the Shandi Lab.	500
Stationary & Consumables	General research supplies, notebooks, archiving materials, and administrative consumables required during data collection.	5000
Immunohistochemistry (IHC) Reagents	Cost of the Zytomed™ IHC kit, primary anti-HER2 antibodies, visualization reagents, chromogen (DAB), and controls for staining all tissue sections.	1500
Molecular Profiling (PCR/ISH)	Cost for advanced molecular studies, including specialized DNA extraction kits for FFPE, PCR primers/probes, or ISH reagents (e.g., HER2/CEP17 probes) and laboratory service fees at Shandi Lab.	2500
Statistical Analysis	Licensing fees for statistical software (e.g., SPSS) or consultation fees for advanced inferential statistical analysis.	300
Printing & Dissemination	Printing and binding of the final thesis, publication costs, and printing of interim progress reports.	100
PhD tuition (flat):	Study and supervision for the whole PhD period	1,000

TOTAL ESTIMATED BUDGET		**11,900 $**

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Appendix

Appendix – 1:

Informed Consent Form

Title of Study:

The Prevalence and Characterization of Human Epidermal Growth Factor Receptor 2 (HER2) Expression Among Colorectal Cancer Patients in Sudan (2025)

Purpose of the Study:

You are invited to participate in a research study aimed at understanding the expression of HER2 in colorectal cancer and its relationship with disease characteristics.

Procedures:

This study will use archived tissue samples and medical record data. No additional procedures will be performed on you.

Risks and Benefits:

There are no direct risks to participants. The results of this study may help improve understanding and future management of colorectal cancer.

Confidentiality:

All information collected will be kept strictly confidential and used for research purposes only.

Voluntary Participation:

Your participation is voluntary, and you have the right to withdraw at any time without any effect on your medical care.

Consent Statement:

I have read and understood the information above and agree to participate in this study.

Participant Name: _____

Signature: _____

Date: _____

Appendix – 2:

Data Collection Checklist

A. Sociodemographic Data

- ☐ Age
- ☐ Sex
- ☐ Place of residence

B. Clinical Data

- ☐ Clinical presentation
- ☐ Tumor location (colon / rectum)
- ☐ Stage of disease (TNM classification, if available)

C. Histopathological Data

- ☐ Histological type
- ☐ Tumor grade
- ☐ Lymphovascular invasion
- ☐ Perineural invasion

D. Immunohistochemistry (HER2) Data

- ☐ HER2 staining status
- ☐ HER2 scoring (0, 1+, 2+, 3+)
- ☐ Final HER2 interpretation (negative / equivocal / positive)