DOI: 10.7860/JCDR/2026/85308.24030

Research Protocol

Pathology Section

Immunohistochemical Evaluation of Oestrogen and Progesterone Receptors in Colorectal Carcinoma and their Association with Tumour Grade and Stage: A Research Protocol

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ABSTRACT

Introduction: Colorectal cancer is one of the major contributors to the global cancer burden, accounting for a predominant proportion of cancer-related deaths and diagnoses. In India, the rising incidence is often linked to dietary habits and lifestyle changes. While Oestrogen Receptors (ER) and Progesterone Receptors (PR) are well established in hormone-based malignancies, their role in Colorectal Carcinoma (CRC) remains sparse.

Need of the study: With little biomarker-based categorisation, colorectal cancer is becoming a greater health concern in India. Although ER and PR are known to exist in hormone-sensitive tumours, it is yet unknown how they relate to prognosis in colorectal cancer. Since there is a dearth of information on ER/PR expression in colorectal cancer in India, the present study aims to assess its correlation with tumour grade and stage and to investigate its possible use in individualised treatment.

Aim: To assess the immunohistochemical expression of ER and PR in CRC and their correlation with tumour grade and stage.

Materials and Methods: An ambispective observational study will be conducted in Department of Pathology at Jawaharlal Nehru Medical College, Datta Meghe Institute of Higher Education and Research, Wardha, Maharashtra, India, from June 2024 to June 2026. Fifty-five patients from the surgery and oncosurgery units who match the inclusion criteria will be enrolled. Histopathological analysis will be conducted on surgically resected colorectal cancer specimens, accompanied by immunohistochemistry labelling to assess the expression of ER and PR. The standard procedures will be followed for processing tissue, staining with Haematoxylin and Eosin (H&E) and immunohistochemistry. The outcomes will be evaluated against histological criteria and Tumour, Node, Metastasis (TNM) staging. The Chi-square test will be used for statistical analysis. A p-value of <0.05 will be considered statistically significant.

Keywords: Colorectal neoplasm, Histopathology techniques, Immunohistochemistry, Neoplasm staging, Prognosis, Steroid receptors, Tumour biomarker

INTRODUCTION

The CRC has now become a major global health burden, contributing significantly to the morbidity and mortality related to cancer. It has gained in incidence over the past two decades in India, which is now among the top ten countries in its incidence worldwide. The rectum and sigmoid colon are the most common sites [1]. This trend is mainly attributed to various modifiable risk factors like increased body weight, physical inactivity, unhealthy eating habits and tobacco use [2].

Histologically, CRC develops from the lining epithelium of the colon and rectum, which often begins as benign polyps that may undergo malignant transformation [3]. Patients may present with symptoms such as bleeding from the rectum, altered bowel habits, anaemia or abdominal discomfort. Colonoscopy remains the primary diagnostic tool, while Computed Tomography (CT) colonoscopy, Magnetic Resonance Imaging (MRI) (especially for rectal cancer) and Positron Emission Tomography-Computed Tomography (PET-CT) are used for staging and metastatic evaluation [4].

Prognosis is gauged through the TNM classification by American Joint Committee on Cancer (AJCC) 8th edition, taking into account the tumour size, depth of invasion, lymph node involvement, histological grade and lymphovascular invasion [5]. Other features, such as tumour budding and margin configuration, may provide additional prognostic information but are not yet considered standard criteria. However, the recent changes have shifted the emphasis to molecular changes, hormonal and genetic biomarkers [5].

The most common genetic alterations seen in CRC include the mutations in regulatory genes such as Adenomatous Polyposis Coli (APC), Tumor Protein 53 (TP53), Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS), Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA), Microsatellite Instability (MSI) and Mismatch Repair (MMR) mechanisms [6]. These molecular changes play an important role in the progression of tumour and response to therapy. They are now an intrinsic to the classification of CRC [7]. For example, APC mutations disrupt the Wnt/β-catenin signalling pathway, that is essential in the development of tumours in the early stages, while Kirsten rat sarcoma viral oncogene homolog (KRAS) and B-Raf proto-oncogene serine/threonine kinase (BRAF) mutations are associated with a more aggressive disease and its resistance to targeted treatments [8].

Steroid hormones, mainly ER and PR, are extensively studied in reproductive cancers. However, their role in colorectal tumourigenesis is still being elucidated [9]. The expression of both ER and PR has been observed both in benign and malignant colonic tissues, though with distinct patterns, which may influence the tumour behaviour and prognosis. ERβ is considered the prominent isoform in the normal colonic epithelium [9]. However, the expression of ERβ tends to decrease during malignant transformation, while ERα expression seems to increase [10]. Similarly, PR levels vary significantly between the healthy and cancerous tissues, suggesting a potential role in CRC biology [11].

In spite of the growth in international interest, Indian studies that explores on ER and PR expression in CRC using immunohistochemistry remain scant, and there is a clear gap in the understanding of the prognostic and predictive value of ER and PR and their correlation with histological grade and TNM staging.

REVIEW OF LITERATURE

There is a continuous change in the molecular characterisation of CRC, while there is a rising interest in normal receptors and alterations in genes as tools for prognosis and for further treatment prediction [12]. While the foundation of clinical assessment is histological grading and TNM staging, there are recent investigations that have highlighted the complex roles of ER and PR in the development of tumours, metastatic potential and responsiveness to treatment [13]. Early detection, when combined with molecular profiling, is considered to have a vital role in improving patient outcomes and tailoring individualised therapeutic approaches. This study seeks to find the association between clinicopathological parameters and the immunohistochemical expression of ER and PR in CRC [14].

In the present study, immunohistochemical evaluation is performed using ER-EP1 (specific for ERα) and PR-EP2 (specific for PR) clones. This ensures isoform-specific staining and methodological consistency with the literature, allowing precise assessment of receptor distribution and avoiding ambiguity in correlating receptor expression with clinicopathological parameters.

Ditonno I et al., stressed the changing function of ER in the biology of colorectal cancer in their systematic study. They pointed out that ERβ acts as a tumour suppressor by stopping the growth of cells and promoting apoptosis. On the other hand, too much ERα is connected to aggressive disease behaviour and worse outcomes. The scientists also said that the balance of receptor isoforms might affect how likely it is for cancer to spread and how well it responds to treatment. They importantly proposed that the combination of ER/PR profiling with recognised molecular markers, including KRAS, MSI and BRAF, could improve prognostic classification and provide personalised therapy strategies [10].

Abd ElLateef AAE et al., in their study on Immunohistochemistry (IHC) analysis of ER/PR in CRC, reported cytoplasmic positivity for ER in 60% of the cases and for PR in 76.66% of the cases in a cohort of 30 patients. The absence of ER negativity was associated mostly with depth of invasion of tumour and the absence of both ER and PR receptors was correlated with increased risk for progression of the disease. The receptor expression was higher in those cases with greater progression-free survival. However, multivariate analysis failed to reach statistical significance, and therefore, a prognostic role for ER and PR in colorectal cancer is possible but inconclusive [11].

According to Topi G et al., the WNT/β-catenin pathway and impaired epithelial integrity may be the principal determinants of metastatic behaviour [15]. In contrast, Caiazza et al. Reported that ERβ, predominantly expressed in healthy colonic epithelium, demonstrates clear antiproliferative and pro-apoptotic activity and may exert a protective influence against colorectal carcinogenesis. Preservation of ERβ expression has been associated with favourable clinical outcomes, whereas its progressive loss corresponds with tumour advancement and poorer prognosis [16].

The study by Refaat B et al., found that reduced PR expression in colorectal carcinoma is linked to adverse clinicopathological features and higher tumour stage. Similarly, loss of ERβ is normally the dominant estrogen receptor in colonic epithelium and it removes key antiproliferative and pro-apoptotic signals, favouring tumour progression. Preservation of ERβ and PR expression may therefore indicate more favourable tumour biology [9].

Further, ER isoforms were differentially expressed in colonic tissues by Campbell-Thompson M et al., with ERβ being more abundant in healthy mucosa and ERα being more abundant in malignant lesions. Underlying changes in tumour biology during carcinogenesis may be reflected in this shift in receptor expression [17].

Taken together, the literature underscores the importance of distinguishing ER isoforms (ERα vs ERβ) in CRC research. By employing ER-EP1 and PR-EP2 clones, the current study ensures methodological specificity in receptor evaluation. Thus, the study aims to assess the immunohistochemical expression of ER and PR receptors in CRC and to evaluate their correlation with tumour grade and stage.

Study Objectives

Primary objective:

- To explore the prognostic significance of ER and PR expression in relation to clinicopathological features.

Secondary objectives:

- To correlate ER and PR expression with histopathological tumour grade.
- To correlate ER and PR expression with TNM stage.
- To determine the immunohistochemical expression of ER and PR in CRC specimens.

Hypotheses

Null Hypothesis (H_0): ER/PR expression will not be significantly associated with adverse clinicopathological features or with poorer outcomes.

Alternate Hypothesis (H_1): ER positivity and PR loss will be significantly associated with higher grade, advanced TNM stage and poorer outcomes. While, ER positivity will also be associated with favourable prognostic features and improved survival.

MATERIALS AND METHODS

The present ambispective observational study will be conducted in Department of Pathology at Jawaharlal Nehru Medical College, Datta Meghe Institute of Higher Education and Research, Wardha, Maharashtra, India, from June 2024 to June 2026. The study has been registered with ClinicalTrials.gov (CTRI/2024/05/068054) and has received ethical clearance from the Institutional Ethics Committee of Datta Meghe Institute of Higher Education and Research (Approval No. DMIHER(DU)/IEC/2024/144). In accordance with the principles of the Declaration of Helsinki, all participants in the prospective phase will provide written informed consent

Inclusion criteria:

- Histopathologically verified diagnosis of CRC;
- Newly diagnosed CRC at first presentation;
- First-time onset CRC with no prior history of cancer or recurrence;
- Patients who have undergone or are scheduled for definitive surgeries (colectomy, hemicolectomy, abdominoperineal resection) with adequate lymph node clearance;
- For retrospective cases, documentation of the above criteria in archived medical records.

Exclusion criteria:

- Individuals diagnosed with non-malignant colorectal conditions;
- Patients with other malignancies, including neuroendocrine tumours and Gastrointestinal Stromal Tumours (GIST);
- Cases presenting with recurrent CRC following prior therapeutic intervention;
- Patients who abandoned, interrupted, or failed to adhere to prescribed treatment protocols;
- Cases without histopathological confirmation of CRC;
- For retrospective cases, records lacking adequate documentation of the above criteria.

Sample size calculation: Using the Krejcie and Morgan formula (1970), the appropriate sample size was determined using the following formula [18]:

$S=X^2 NP (1-P)$

$d^2 (N-1) + X^2P (1-P)$

Where,

N=60 (target population)

P=0.5 (p denotes expected prevalence)

d=0.05 (margin of error)

X^2=3.841 (Chi-square value for 1 degree of freedom at 95% confidence level)

S=3.841×60×0.5(1-0.5)/ (0.0025×59) + (3.841×0.25)

S=57.6/0.147+0.960

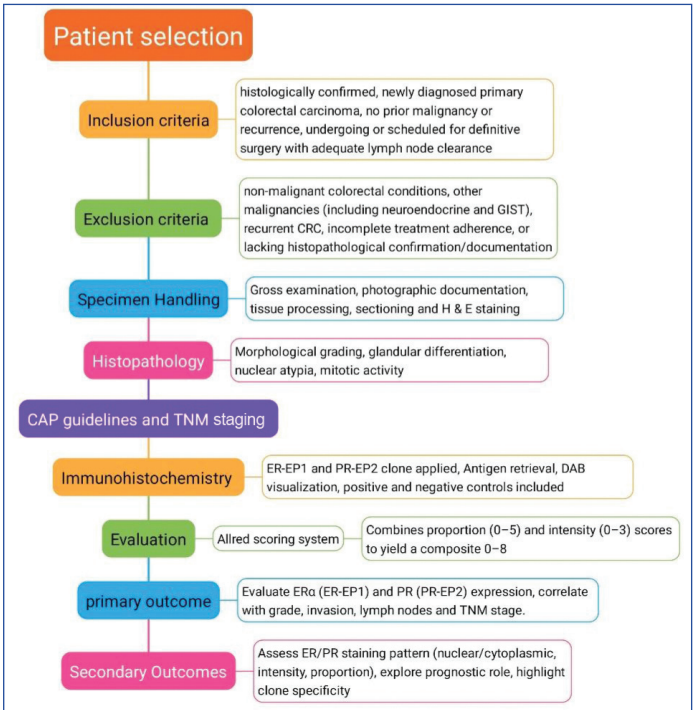
S=57.6/1.107

S=approximately 52.04

Thus, the estimated sample size is approximately 52 cases of CRC. To account for possible exclusions or dropouts, the study will target 55 cases, ensuring statistical validity and comparability with prior research.

Study Procedure

The participants' demographic details, such as name, age, gender, registration number, clinical unit and the department from which referred Outpatient Department (OPD) and Inpatient Department (IPD), will be recorded [Table/Fig-1].



[Table/Fig-1]: Flowchart of research work.

The gross examination of colorectal specimens will be performed by carefully describing the anatomical landmarks, documenting any visible pathological changes, and locating lymph nodes. Photographic documentation of the gross specimen will be captured and representative tissue sections, including tumour sites and all lymph nodes, will be submitted for microscopic examination [19].

Routine histopathology will be carried out using the H&E staining protocol. Tissue sections will be treated in three consecutive xylene baths for ten minutes each, followed by passage through descending grades of alcohol and immersion in water. Harris haematoxylin will be applied for ten minutes to stain the nuclei, after which the sections will be rinsed, treated with 1% acid alcohol, and blued in alkaline tap water. Cytoplasmic components will be stained with 1% aqueous eosin for one minute. The slides will then be dehydrated in 90% alcohol and mounted using Dibutylphthalate Polystyrene Xylene (DPX) [19,20].

For systematic documentation, grading will be recorded with explicit mention of the percentage of gland formation, the degree of nuclear atypia and the quantified mitotic figures per high power field. This structured approach ensures reproducibility and allows correlation with other pathological parameters such as depth of invasion, lymphovascular invasion, tumour budding and special histological variants like mucinous or signet-ring carcinoma [21,22].

Immunohistochemical staining will be performed on 4 µm thick sections from formalin-fixed, paraffin-embedded CRC tissue blocks mounted on charged slides and incubated at 60-70°C for one hour, followed by deparaffinisation in xylene and rehydration through graded alcohols to distilled water. Antigen retrieval will be carried out by microwave heating in 10 mM citrate buffer (pH 6.0) for 15-20 minutes, followed by cooling and rinsing in Tris-buffered saline. Endogenous peroxidase activity will be blocked using 0.5% hydrogen peroxide for 5-10 minutes.

Primary antibodies ER (ERα, Clone EP1, Mouse Monoclonal, Dako/Agilent) and PR (PR, Clone EP2, Rabbit Monoclonal, Thermo Fisher Scientific) will be applied at optimised dilutions (1:100) and incubated overnight at 4°C. A peroxidase-linked secondary antibody system will then be applied, and immunoreactivity will be visualised using DAB chromogen. Slides will be counterstained with haematoxylin, dehydrated, cleared in xylene, and mounted with DPX. Endometrial tissue will serve as positive control, while negative controls will be prepared by substituting the primary antibody with buffer. Stained slides will be examined under light microscopy at magnifications ranging from 40× to 400×, and receptor expression will be evaluated using the Allred scoring system [23,24].

Study outcomes

Primary outcomes: Histological grading of CRC is based on morphological criteria [19]:

- Glandular differentiation;
- Nuclear atypia;
- Mitotic activity.

Conventional three-tier system:

- Well differentiated;
- Moderately differentiated;
- Poorly differentiated.

AJCC two-tier system:

- **Low-grade:** well and moderately differentiated tumours;
- **High-grade:** poorly differentiated and undifferentiated tumours.

Immunohistochemical Expression of ERα and PR: Immunohistochemical expression of ER and PR in CRC will be assessed using the Allred scoring system (score 0-8) [25] based on staining intensity and proportion. The scoring system includes a proportion score (0-5) based on the percentage of positively stained tumour cells. An intensity score (0-3) is assigned according to the strength of immunostaining. The composite score (0-8) is obtained by summing the proportion and intensity scores.

Score interpretation:

- **0-1:** Negative (undetectable expression)
- **2-3:** Weak/faint positivity
- **4-6:** Moderate expression
- **7-8:** Strong immunoreactivity

The Allred Scoring System, although originally developed for breast cancer pathology, can be applied in CRC research to evaluate

immunohistochemical staining of biomarkers such as ER and PR [25]. When applied to CRC, this scoring system provides a reproducible and semi-quantitative approach for assessing hormone receptor status, enabling correlation with tumour grade, TNM stage, and other pathological parameters [26].

Secondary outcomes

Detailed ER/PR staining pattern: nuclear vs. cytoplasmic localisation, intensity, and proportion.

Prognostic role: Explore association of ER/PR expression with tumour budding, lymphovascular invasion, and special histological variants (e.g., mucinous, signet-ring carcinoma).

Clone specificity: Assess reproducibility and reliability of ER-EP1 and PR-EP2 clones in CRC.

STATISTICAL ANALYSIS

Data will be analysed using IBM Statistical Package for the Social Sciences (SPSS) Statistics software version 25.0. Qualitative variables will be expressed numerically and described as percentages. Comparisons between groups will be performed using the Chi-square test for categorical variables. Hazard ratios for each covariate against a baseline category will be calculated, along with their corresponding p-values and 95% Confidence Intervals (CIs), to assess prognostic significance. A p-value <0.05 will be considered statistically significant.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]

- Plagiarism X-checker: Dec 04, 2025
- Manual Googling: Apr 02, 2026
- iThenticate Software: Apr 04, 2026 (3%)

ETYMOLOGY: Author Origin

EMENDATIONS: 7

AUTHOR DECLARATION:

- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Nov 23, 2025**
Date of Peer Review: **Dec 06, 2025**
Date of Acceptance: **Apr 06, 2026**
Date of Publishing: **Aug 01, 2026**