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Molecular Classification Trail of Colorectal Cancer Using Immunohistochemical Markers

Amany Mahmoud Mohamed[1], Sabah Ahmed Mohamed Fadel[2], Mohamed Mostafa Ali Wahdan[3], Abdallah Hedia Mohamed[4], Mahmoud Gamal Ameen Taher[5]

[1]Assistant lecturer, Pathology Department, Faculty of Medicine, South Valley University

[2]Professor of Pathology, Head of the Department of Pathology, Faculty of Medicine, Quena University

[3]Assistant Professor of Clinical Oncology, Faculty of Medicine, Quena University

[4]Lecturer of Clinical Oncology, South Egypt Cancer Institute, Assiut University

[5]Assistant Professor of the Oncological Pathology department, South Egypt Cancer Institute, Assiut University

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ABSTRACT

Colorectal cancer (CRC), the third most common and second deadliest malignancy worldwide, is primarily composed of adenocarcinomas and exhibits notable genetic, molecular, and histopathological heterogeneity. Its progression involves chromosomal instability, microsatellite instability, and CpG island methylation, with major genetic contributors including APC, TP53, and KRAS mutations. Risk factors range from hereditary syndromes and inflammatory bowel diseases to dietary and environmental exposures. CRC is classified into four consensus molecular subtypes (CMS): CMS1 (hypermutated, immune-rich), CMS2 (epithelial, chromosomally unstable), CMS3 (epithelial, KRAS-mutated, metabolic), and CMS4 (mesenchymal, invasive, with poor prognosis). Histologically, CRC tumors exhibit features such as tumor budding, tumor-infiltrating lymphocytes (TILs), tumor deposits (TDs), perineural invasion (PNI), and lymphovascular invasion (LVI), all of which impact prognosis and therapeutic response. The epithelial-mesenchymal transition (EMT) plays a crucial role in tumor progression, metastasis, and resistance, marked by loss of E-cadherin and nuclear β-catenin localization alongside vimentin expression. Immunohistochemistry (IHC) aids in molecular classification using markers such as CDX2, CK20, CK7, β-catenin, vimentin, and E-cadherin. High vimentin and nuclear β-catenin correlate with poor survival, while E-cadherin loss predicts invasiveness and poor outcomes. EMT-regulated gene signatures and tumor microenvironment components, particularly cancer-associated fibroblasts and macrophages, further influence tumor behavior and therapeutic resistance. Accurate subtyping using IHC panels provides a cost-effective alternative to transcriptomic profiling, enhancing prognostication and guiding individualized therapy. Integrating molecular, histological, and immunohistochemical features strengthens CRC classification, enabling more precise staging, prognostication, and treatment planning. The review aims to demonstrate how immunohistochemical markers can be used to classify colorectal cancer into molecular subtypes for improved diagnosis, prognosis, and personalized treatment

Keywords: *Colorectal Cancer, Molecular Classification, Immunohistochemistry, Biomarkers, Tumor Subtypes.*

1. INTRODUCTION

Colorectal cancer (CRC) ranks third in incidence (6.1%) and second in mortality (9.2%). Over 90% are adenocarcinomas originating from epithelial colorectal mucosa cells, the most common type affecting the colon and rectum. CRC exhibits strong familial inheritance, with increased risks linked to personal or family cancer history, colon polyps, inflammatory bowel disease, diabetes mellitus, or cholecystectomy [1].

Recognized adenocarcinoma subtypes include common forms like adenoma-like, mucinous, micropapillary, signet ring, undifferentiated, and rare types such as adenosquamous carcinoma with sarcomatoid and medullary components. Uncommon subtypes are clear cell adenocarcinoma, low-grade tubuloglandular adenocarcinoma, and villous/adenoma-like adenocarcinoma [2].

Favorable prognostic markers in colon adenocarcinoma include microsatellite instability and increased tumor-infiltrating lymphocytes [3]. Immunohistochemical markers commonly used to confirm colorectal adenocarcinoma origin in metastatic disease are cytokeratin CK20 and CDX2 positivity, with CK7 negativity [4].

Epithelial cells express membrane adhesion proteins like E-cadherin and β-catenin. Loss of E-cadherin expression, nuclear

translocation of β-catenin, and increased expression of mesenchymal markers such as Slug, Twist, or vimentin characterize mesenchymal phenotypes [5].

CRC molecularly classifies into four main consensus molecular subtypes (CMS): CMS1 includes hypermutated tumors with BRAF mutations, microsatellite instability, and prominent immune reactions; CMS2 is an epithelial subtype with activated WNT/MYC signaling and chromosomal instability [6]; CMS3, also epithelial, is characterized by KRAS mutations and metabolic changes [7]; CMS4 is a mesenchymal subtype with transforming growth factor-β activation, stromal invasion, angiogenesis, poor prognosis, increased metastasis risk, and chemoresistance. Tumors exhibiting both epithelial and mesenchymal features are considered transition phenotypes [8].

CRC is categorized as epithelial if tumors are positive for membrane E-cadherin and β-catenin without vimentin expression. Mesenchymal CRC lacks E-cadherin, demonstrates β-catenin nuclear localization, and expresses vimentin. Transition phenotypes, typically epithelial in the tumor core and mesenchymal at invasive fronts, are classified within mesenchymal CRC [9].

Vimentin, a multifunctional intermediate filament protein, regulates various cellular functions. E-cadherin, a classical epithelial marker belonging to the cadherin family of transmembrane glycoproteins, maintains cell adhesion and tissue structure. β-catenin, a 92-kDa protein associated with E-cadherin, also independently participates in the Wnt signaling pathway. Mutations in APC complex components elevate cytoplasmic β-catenin, prompting its nuclear translocation, transcription factor activity via binding TCF/LEF, activation of target genes (CyclinD1, c-Myc, CD44, Survivin), and consequent uncontrolled proliferation in CRC cells [10]

2. HISTOLOGY OF COLON

Colon Compartments

Histologically, the colon comprises mucosa, submucosa, muscularis propria, and serosa (Fig 1, 2) **[11].**

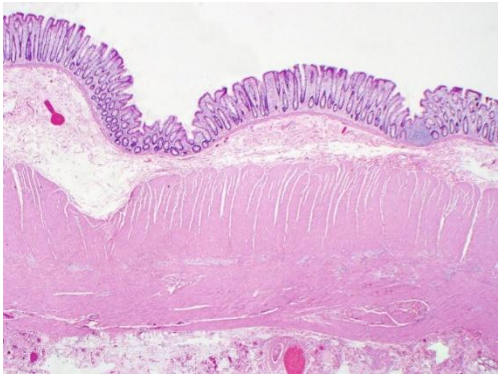

Figure (1): Full-thickness section of colon showing four layers: mucosa, submucosa, muscularis propria, and serosa [11].

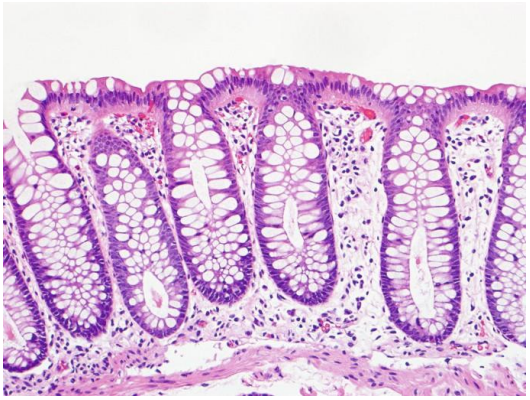

Figure (2): Normal colonic mucosa. Regularly and parallelly arranged crypts perpendicular to the muscularis mucosae consisting of absorptive cells, goblet cells, and endocrine cells. Paneth cells are normally present in proximal colon. The lamina propria contains a variable number of inflammatory cells and a rich network of capillaries, venules, and lymphatics [11].

Mucosa

The mucosa consists of columnar epithelium lining colonic crypts, arranged perpendicularly, resembling a "rack of test tubes," extending into the lamina propria to muscularis mucosae. Epithelial cells include absorptive, goblet, endocrine, and Paneth cells. The basement membrane is typically 3–5 μm thick; pathological irregularity (>10 μm) involves capillary entrapment **[11].**Absorptive cells predominate, characterized by columnar shape, eosinophilic cytoplasm, basal oval nuclei, and dense microvilli. Goblet cells appear with clear cytoplasm, dense irregular nuclei, found in surface and crypt epithelium. Endocrine cells in crypts have small, deeply eosinophilic granules, basal nuclei displaced luminally. Paneth cells are pyramidal, located at crypt bases, primarily in the proximal colon, with eosinophilic autofluorescent granules **[11].**

Lamina Propria

The lamina propria connects the basement membrane and muscularis mucosae, comprising inflammatory and mesenchymal cells within extracellular matrix. It contains numerous lymphoid aggregates, particularly in the cecum, along with lymphocytes, plasma cells, eosinophils, mast cells, macrophages, and mucin-ingesting muciphages, more abundant in the left colon and rectum **[12].** Cellular density decreases from the right to left colon. It also includes extensive vascular and lymphatic networks and nerve fibers. The muscularis mucosae forms its deep boundary **[11].**

Submucosa

Submucosa contains loose smooth muscle, fibroadipose tissue, angiolymphatic and neural networks, scattered inflammatory cells, and occasional lymphoid aggregates. Adipose tissue varies by location, abundant around ileocecal valve and cecum. Neural elements include Meissner plexus (beneath muscularis mucosae) and deeper Henle's plexus, comprising neurons, glial cells, and ganglion cells. Interstitial cells of Cajal regulate gut motility **[11].**

Muscularis Propria and Serosa

Muscularis propria includes inner circular and outer longitudinal muscle layers, interspersed blood vessels, lymphatics, and interstitial cells of Cajal. The serosa, composed of mesothelial lining and fibroelastic tissue, bounds the muscularis propria externally **[11].**

Historical Background of Colorectal Cancer (CRC)

Molecular Pathogenesis

CRC is multifactorial, progressing from epithelial hyperplasia, atypical hyperplasia, adenoma, carcinoma in situ to invasive carcinoma due to DNA alterations. Three molecular mechanisms are established: chromosomal instability (common in Familial Adenomatous Polyposis (FAP)) **[13]**, genetic mutations including Lynch syndrome and mismatch repair (MMR) defects **[14],** and CpG island hypermethylation in gene promoters **[15].** Frequent genetic alterations involve APC, DCC, P53, K-Ras, c-MYC, MCC, and MMR genes (hMLH1, hMLH3, hMSH2, hMSH3, hMSH6, hPMS1, hPMS2). Multiple abnormal pathways often coexist **[16].**

Risk Factors

CRC etiology is uncertain, but several factors contribute:

- **Genetic:** 20% of CRCs have a genetic basis, with threefold increased risk in first-degree relatives. Syndromes include FAP and MMR-related inherited CRC **[17].**
- **Dietary:** High-fat, high-animal protein, low-fiber diets promote CRC through increased bile acid secretion and intestinal carcinogens **[18].**
- **Non-cancerous diseases:** Ulcerative colitis (UC), Crohn's disease, polyps, and adenomas increase CRC risk. CRC develops in 3-5% of UC patients, rising to over 10% after 20 years. Colonic polyps precede 15-40% of cancers; adenomas <1 cm have <2% cancer risk, while >3 cm adenomas exceed 40% risk **[19].**
- **Other factors:** Carcinogen exposure, sedentary lifestyle, obesity, and pelvic radiation elevate sigmoid and rectal cancer risk **[20].**

Molecular Classification

CRC exhibits two major genomic instability patterns: chromosomal instability (CIN) and microsatellite instability (MSI). CIN, involving somatic copy number alterations, accounts for ~84% of sporadic CRCs, frequently linked to APC and TP53 mutations **[21].** MSI, present in ~13-16% of cases, is due to defective MMR, associated with wild-type TP53, diploid chromosomal pattern, and CpG island methylation phenotype (CIMP), notably silencing MLH1 **[22, 23]**. The Cancer Genome Atlas **[24]** supports this DNA-based division into MSI (~13-16%) and CIN (~84%).

At the transcriptomic level, CRC has four Consensus Molecular Subtypes (CMS): CMS1 (hypermutated, BRAF mutations,

MSI, immune involvement), CMS2 (epithelial, chromosomally unstable, WNT and MYC activated), CMS3 (epithelial, KRAS mutations, metabolic changes), and CMS4 (mesenchymal, TGF-β activation, stromal invasion, poor prognosis, metastasis, chemoresistance) **[25, 26].**

Tumor Deposits in Colorectal Cancer (CRC)

Definition and Significance

Tumor deposits (TDs) are isolated tumor clusters in pericolonic or perirectal fat within the lymphatic drainage area, without identifiable lymph node structures, arising from discontinuous or vascular/perineural spread **[27]**. TDs occur in approximately 20% of CRC cases, negatively affecting prognosis **[28].** The 8th edition of TNM classifies TDs without lymph node metastasis as N1c; however, recent studies suggest TDs independently predict prognosis regardless of nodal status **[29, 30]** Caution is advised in post-neoadjuvant settings, as isolated tumor cells might represent residual viable tumor unrelated to typical TDs **[27, 31].**

Prognostic Impact

A meta-analysis of 17 studies (10,106 patients) showed a 22% TD incidence (range 5%-42%) in stage I-IV CRC. TD presence negatively impacted prognosis, with hazard ratios (HR) of 2.2 for disease-free survival, 3.3 for disease-specific survival, and 2.9 for overall survival. Multivariate analyses supported TDs as an independent prognostic marker **[32].** The current N1c category is inadequate; thus, TDs and their number should be incorporated into N staging **[33].**

Tumor-Infiltrating Lymphocytes (TILs)

Role and Prognostic Value

TILs respond to tumor-associated antigens presented by antigen-presenting cells, affecting tumor invasion through angiogenesis, apoptosis, and macrophage activation **[34]** Increased CD3/CD8 ratios indicate better prognosis and chemoradiotherapy responses in rectal cancer **[35].** High FoxP3+ T-lymphocyte infiltration is favorable in colon cancer patients undergoing chemotherapy or immunotherapy but correlates with higher lymph node metastasis risk when abundant at invasive fronts **[36]**. Low TIL infiltration correlates with advanced stage and poor prognosis, emphasizing their inclusion in pathology reports **[37].**

Immunoscore and Clinical Application

The "Immunoscore," quantifying CD3+ and CD8+ lymphocytes at tumor centers and invasive margins, predicts recurrence better than traditional TNM staging, validating its clinical utility **[38].**

Tumor Budding

Definition and Prevalence

Tumor budding involves single cells or small clusters found intratumorally (ITB) or peritumorally (PTB), occurring in approximately 40% of CRC cases **[39, 40].** It reflects aggressive tumor behavior and poor prognosis, warranting its inclusion in clinical pathology practice per ITBCC guidelines **[41, 42].**

Clinical Implications

Clinically, PTB aids surgical decision-making in pT1 CRC, guides adjuvant therapy considerations in stage II CRC (**Table 1**), and influences preoperative planning in rectal cancer via biopsy-detected ITB. Additionally, tumor budding in metastatic CRC assists patient stratification for treatments. Its utility in stage III CRC for predicting chemotherapy response remains unclear

Table (1): Clinical scenarios of tumor budding in colorectal cancer [43].

Clinical scenario	ITBCC recommendations	Clinical implication	Prognostic value	Predictive value
pT1 CRC	Tumor budding is an independent predictor of lymph node metastasis in pT1 CRC	Oncologic resection	Yes	Unclear, validation needed
Stage II CRC	Tumor budding is an independent predictor of survival in stage II CRC	Adjuvant therapy	Yes	Unclear, validation needed

ITB in preoperative biopsies	ITB in CRC has been shown to be related to lymph node metastasis	Neoadjuvant therapy in rectal cancer Preoperative surgical management in colon cancer	Yes	Possible, validation needed
Tumor budding CRLM	No recommendation	Additional factor for management of stage IV CRC patients	Possible, validation needed	Unclear, validation needed

Epithelial-Mesenchymal Transition (EMT)

Tumor budding reflects EMT, a key process enhancing tumor aggression and metastasis potential. Tumor budding independently predicts recurrence risk in stage III-IV CRC and should be included in standardized pathology reports for effective risk stratification **[44, 45].** Higher tumor budding counts (≥10) indicate greater absolute benefit from chemotherapy regarding recurrence and mortality reduction **[46].**

Perineural Invasion (PNI)

Definition and Significance

PNI involves tumor infiltration in and around nerves. Originally defined as nerve invasion **[47],** a refined definition includes tumor proximity involving ≥33% of nerve circumference or invasion of nerve sheath layers **[48].** PNI frequency in colorectal cancer (CRC) ranges from 9–33%, increasing with stage: 10% in stages I-II, 30% in stage III, and 40% in stage IV **[49-51]**. PNI correlates with poor outcomes, rapid progression, larger tumor size, deeper invasion, lymph node involvement, poor differentiation, and metastases **[52, 53]**. It's recognized as a negative prognostic factor in TNM staging (8th edition) and pathology reporting **[54].**

Clinical Implications

PNI negatively affects metastasis, recurrence, overall survival (OS), and disease-free survival (DFS). Adjuvant chemotherapy improves 5-year DFS and mitigates PNI's adverse prognosis in stage II-III CRC, making PNI useful for selecting patients for adjuvant therapy **[53, 55].**

Lymphovascular Emboli (LVI)

Prognostic Impact

LVI independently predicts poor 5-year OS and DFS, indicating aggressive tumor behavior in CRC **[56].** It's significant for predicting lymph node micrometastases, especially in AJCC stage II CRC. Incorporating LVI with the 8th TNM system improves prognostic accuracy **[57-59].**

Clinical Utility

LVI presence supports identifying high-risk CRC patients and is valuable in survival nomograms, enhancing prognostic accuracy for individual patient management **[59].**

Epithelial-Mesenchymal Transition (EMT)

Definition and Mechanism

EMT is a reversible biological process where epithelial cells acquire mesenchymal traits, facilitating tumor cell migration, invasion, metastasis, and resistance to therapy **[44, 60]** Over 90% of CRC lines exhibit partial EMT, contributing to metastasis and tumor initiation **[61].** EMT is regulated transcriptionally (Snail, Twist, Zeb), post-translationally, epigenetically, and via non-coding RNA pathways. EMT transcription factors like SNAIL, TWIST1, and ZEB1 directly repress epithelial genes and enhance mesenchymal traits **[62, 63].** Specifically, SNAIL suppresses E-cadherin expression via WNT signaling and regulates IL-8, promoting tumor stemness **[64, 65].** TWIST1 and ZEB1 expressions correlate with enhanced invasiveness and metastasis in CRC **[66, 67].**

Clinical Significance

EMT strongly predicts poor prognosis, metastasis, and chemoresistance. EMT activation reduces sensitivity to anti-EGFR therapy in metastatic CRC **[68],** particularly in right-sided tumors, due to increased TGF-α, AXL, EPHA2, and TGF-β signaling **[69]** EMT indicators predict outcomes and represent potential therapeutic targets. Combining EMT inhibitors with chemotherapy or using EMT markers in adjuvant therapy may prevent relapse **[70].**

Molecular Subtypes and Prognostic Models

EMT-related gene signatures (e.g., CXCL1, PCOLCE2) show prognostic potential in CRC subtyping and patient risk assessment **[71].** Prognostic models based on EMT-related genes offer improved prediction of recurrence in stages II/III CRC **[72, 73].** EMT signatures combined with pathological staging enhance survival prediction and correlate with immune cell infiltration in CRC microenvironments **[74].**

Tumor Microenvironment (TME)

Mesenchymal gene expression in CRC transcriptomes predominantly originates from TME stromal cells like cancer-associated fibroblasts **[75].** Tumor-associated macrophages (TAMs) facilitate EMT via the JAK2/STAT3/miR-506-3p/FoxQ1 axis, enhancing metastatic spread **[76].** Notch and WNT pathways further regulate EMT, stemness, and invasive behavior in CRC **[73].** Increased miR-4775 expression promotes EMT through TGF-β signaling, driving metastasis **[77].**

Molecular Classification and Immunohistochemistry in Colorectal Cancer (CRC)

CMS Immunohistochemical Classification

Trinh et al. **[78]** introduced a practical immunohistochemical (IHC) panel with five markers (FRMD6, ZEB1, HTR2B, CDX2, cytokeratin), achieving 87% concordance with transcriptomic Consensus Molecular Subtypes (CMS). MSI/dMMR defined CMS1, while the four remaining markers classified MSS cancers into epithelial (CMS2/3 combined) or mesenchymal (CMS4) subtypes, despite limitations in differentiating CMS2 and CMS3. Intratumor heterogeneity and non-standardized biopsies affect classification accuracy. A similar "Mini Classifier" with four markers (FRMD6, ZEB1, HTR2B, CDX2) plus cytokeratin provides accurate CRC subtype identification (Fig 3, 4) **[79].**

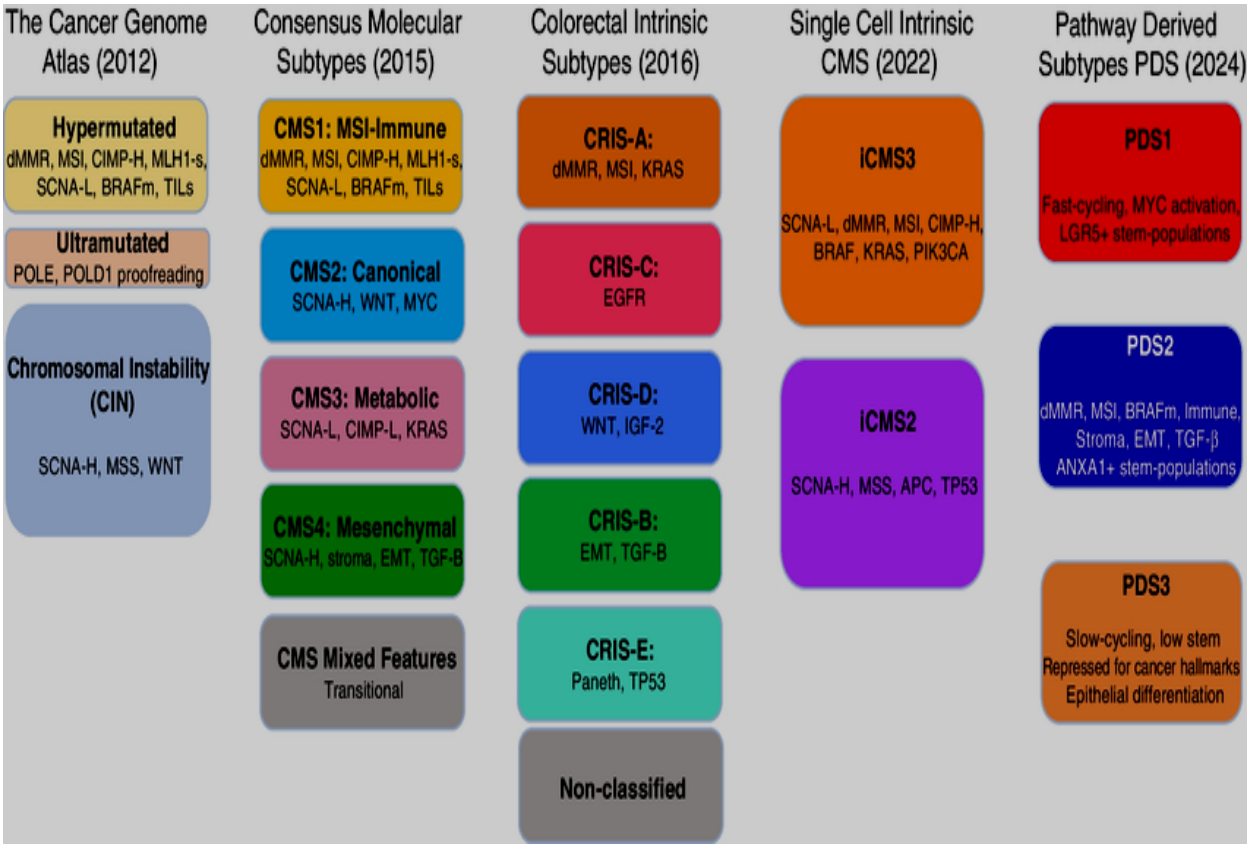

Figure (3): Diagrammatic summary of colorectal cancer molecular pathology classification systems [8].

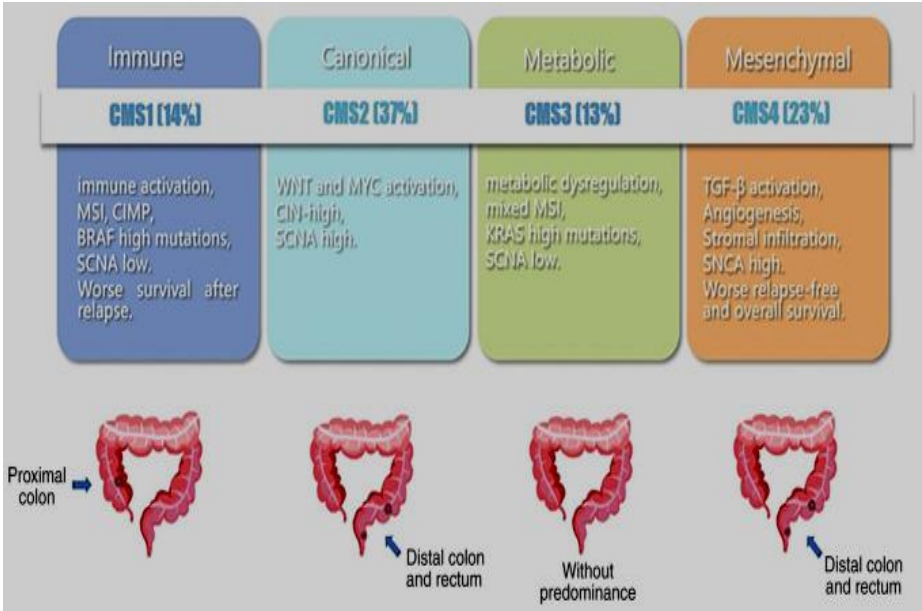

Figure (4): Main characteristics of CMS classification in colorectal cancer [80].

Vimentin

Role and Prognostic Significance

Vimentin, an intermediate filament protein expressed during epithelial-to-mesenchymal transition (EMT), facilitates cell migration and metastasis **[81, 82].** High vimentin expression at CRC's invasive front correlates with poor overall survival (OS), progression, and higher tumor grade (G3 vs. G1) **[83, 84].** Computational models identify vimentin as a promising CRC biomarker **[85].** Additionally, vimentin gene methylation serves as a sensitive marker for advanced CRC in serum/stool samples (Fig 5) **[86].**

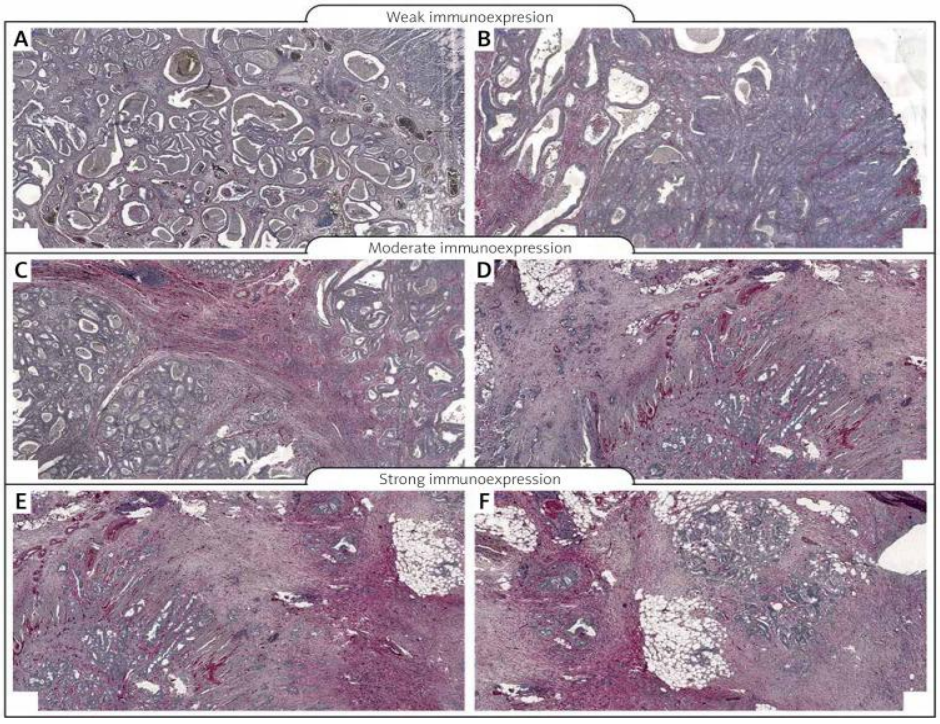

Figure (5): Immunohistochemical expression of vimentin protein in tumor tissue: A, B – weak expression of vimentin protein in colon adenocarcinoma samples, C, D – moderate expression of vimentin; E, F – strong expression of vimentin [84].

β-catenin

Wnt/β-catenin Pathway in CRC

Wnt signaling (canonical, β-catenin-dependent; non-canonical) is crucial in CRC pathogenesis. Canonical Wnt/β-catenin signaling controls proliferation, differentiation, migration, and gene expression **[87]**. affecting this pathway are common in CRC: 70–80% involve APC gene truncation, reducing β-catenin degradation, while gain-of-function β-catenin mutations occur in about 50% of CRC cases without APC mutations **[88, 89].** Elevated nuclear β-catenin expression significantly predicts poorer disease-free survival (DFS), cancer-specific survival (CSS), and OS, while low membranous β-catenin also correlates with poor OS **[90].**

Clinical and Therapeutic Implications

β-catenin regulates metastasis-promoting genes such as S100A4. Elevated S100A4 expression correlates with reduced OS and metastasis-free survival (MFS) in CRC. Therapeutic interventions targeting Wnt signaling and β-catenin transcriptional activity could inhibit metastatic CRC **[88]** Moreover, β-catenin/TCF interaction inhibitors and transcriptional co-activator antagonists represent viable therapeutic strategies **[87]**.

E-cadherin

Structure and Function

E-cadherin, encoded by CDH1, is a calcium-dependent adhesion glycoprotein linking epithelial cells via cadherin-catenin complexes **[91].** Its downregulation, a hallmark of EMT, disrupts epithelial polarity, facilitating tumor invasion and metastasis **[92, 93]** E-cadherin inactivation in cancer results from mutations, epigenetic silencing, increased endocytosis, and proteolysis **[91].**

Prognostic Value in CRC

Low E-cadherin expression (found in ~60% of CRC cases) correlates with advanced T stage, lymph node involvement, metastasis, higher TNM stage, and poor 5-year survival rates, marking it as an independent prognostic indicator **[94, 95].** Incorporating E-cadherin expression assessment alongside standard TNM classification enhances prognostic accuracy in CRC staging **[96]**

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