Molecular Classification of Colorectal Cancer

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Molecular Classification

- Biological classification of tumors


- Reflects common biological features useful for diagnosis, prognosis and therapy. Some with predictive value

- Allows personalized targeted therapy
Molecular alterations in CRC

**Genetic Alterations**
- Chromosomal Instability (CIN)
  - MAPK pathway activation
  - RAS, B-RAF mutations
- Microsatellite Instability (MSI)
- CpG island mutator phenotype (CIMP)
  - Aberrant methylation of tumor suppressor genes

**Epigenetic Alterations**
- Aberrant methylation of tumor suppressor genes

**Sporadic**
- Constitutional mosaic Epimutation (MLH1)
  - FAP; AFAP
  - Mixed Polyposis Syndrome
  - Ashkenazi I1307K
  - CHEK2 (HBCC)
  - MUTYH (MAP)
  - TGFBR1

**Hereditary**
- Lynch Syndrome
- AC-1 without MMR
  (Familial CRC of syndrome “X”)
- TACSTD1 (EPCAM)

= as yet undiscovered hereditary cancer variants

HT Lynch, 2013
CIN pathway of CCR

Normal → Mucosa at risk → Aberrant crypt foci → Tubular Adenoma → High-grade Dysplasia → Adenocarcinoma

Germ line/aquired somatic “first hit” APC

Methylation / activation “2nd hit” APC/b-catenin

Protooncogene mut K-RAS CIN or MSI

Homozygous loss suppressor genes p53, LOH18q, SMAD2,4,↑COX2

Additional mut, gross chromosome alt, telomerase, ↑genes

Progressive accumulation of mutations (APC, RAS, p53, etc...)
Practical uses of CRC molecular pathology

• Therapy guidance
  – Targets
    • RAS
    – Microsatellite instability
    • BRAF

• Identification of patients at risk
  – Lynch Syndrome
  – MSI
Molecular pathways in CRC

- Wnt pathway
- EGFR pathway
- HER2 pathway
- IGF pathway
- VEGF pathway

- Ras
- PI3K
- PTEN
- AKT
- mTOR

- APC
- Raf
- MEK
- ERK
- B-catenin
- TCF
- MYC
PUTATIVE MOLECULAR PATHWAYS TO COLORECTAL CARCINOMA

Serrated pathways

Normal mucosa

BRAF CIMP-H

SSA

MLH1 loss

SSAD (frameshift mutations e.g. TGFrIII IGFIIIR)

BRAF CIMP-H

MSI CRC

MSS CRC

Barbra

Resistant to 5FU

Resistant to anti-EGFR therapy

Familial pathways

Lynch (germline mutation of a MMR gene)

APC

Loss of remaining MMR allele, p53

Wnt

Loss of remaining APC allele

Hypomethylation

TSA + sTVA

Hundreds of TAs

TA

MSI (frameshift mutations e.g. TGFrIII IGFIIIR)

CIMP-MSS CRC

SMAD4, p53

KRAS

Normal mucosa

CIMP-MSS CRC

KRAS, CIMP-L MSS CRC

Good prognosis

Sensitive to 5FU

Sensitive to anti-EGFR therapy

Poor prognosis

Sensitive to 5FU

Resistant to anti-EGFR therapy

Conventional pathways

FAP (germline mutation of APC gene)

APC

Loss of remaining APC allele

Hypomethylation

TVA

Thousands of TAs

TA

TA HGD

MSI (frameshift mutations e.g. TGFrIII IGFIIIR)

CIMP-MSS CRC

SMAD4, p53

KRAS

Normal mucosa

Good prognosis

Sensitive to 5FU

Sensitive to anti-EGFR therapy

Poor prognosis

Resistant to 5FU

Resistant to anti-EGFR therapy

Good prognosis

Sensitive to 5FU

Sensitive to anti-EGFR therapy

Poor prognosis

Resistant to 5FU

Resistant to anti-EGFR therapy

Tumors are heterogeneous

A Heterogenous Tumor

Molecular Cell, Vol 5. 2014
What sample do we need?

DOI 10.1007/s00280-010-1298-9

SHORT COMMUNICATION

Acquired KRAS mutations during progression of colorectal cancer metastases: possible implications for therapy and prognosis

Mohamed Bouchahda · Abdoulaye Karaboué · Raphaël Saffroy · Pasquale Innominato · Lee Gorden · Catherine Guettier · René Adam · Francis Lévi

<table>
<thead>
<tr>
<th>Type and date of surgery</th>
<th>Sample origin</th>
<th>Proportion of tumor cells (%)</th>
<th>KRAS status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segmental colectomy OCT, 2004</td>
<td>Primary colon</td>
<td>40</td>
<td>wt</td>
</tr>
<tr>
<td>First hepatectomy AUG 2005</td>
<td>Synchronous liver metastasis (lesion # 1)</td>
<td>30</td>
<td>wt</td>
</tr>
<tr>
<td></td>
<td>Synchronous liver metastasis (lesion # 2)</td>
<td>55</td>
<td>wt</td>
</tr>
<tr>
<td>Second hepatectomy JUL 2006</td>
<td>Metachronous liver metastasis (lesion # 3)</td>
<td>50</td>
<td>Mutated G38A/G13D</td>
</tr>
<tr>
<td></td>
<td>Metachronous liver metastasis (lesion # 4)</td>
<td>40</td>
<td>Mutated G38A/G12D</td>
</tr>
</tbody>
</table>
What sample do we select?

**Synchronous tumors:**
- Analyze both tumors separately
- They are independent neoplasms

**Metastatic tumors:**
- Analyze the metastasis

**Multiple evolving metastases:**
- Analyze the most recent metastasis
What method do we use?

- Sanger sequencing
- Pyrosequencing
- K-RAS strip assay
- Next Generation Sequencing (NGS)
Which mutations do we analyze?

**KRAS** mutations
Exon 2 codons 12 and 13

**CONCLUSIONS**

Additional RAS mutations predicted a lack of response in patients who received panitumumab–FOLFOX4. In patients who had metastatic colorectal cancer without RAS mutations, improvements in overall survival were observed with panitumumab–FOLFOX4 therapy. (Funded by Amgen and others; PRIME ClinicalTrials.gov number, NCT00364013.)
PEAK Trial

mFOLFOX6 + Panitumumab or Bevacizumab in 1st-line Treatment of WT KRAS exon 2 mCRC (Open Label, Phase 2)

Metastatic CRC (n=285)

R

1:1

mFOLFOX6 (Q2W) + panitumumab 6 mg/kg (Q2W)

mFOLFOX6 (Q2W) + bevacizumab 5 mg/kg (Q2W)

Tumour Assessment Q8W (±7 days); Treatment administered until disease progression, death, or withdrawal from study

END OF TREATMENT

SAFETY FOLLOW UP

Every 3 months (±28 days) until end of study

END OF STUDY

Study endpoints: PFS* (1°); OS, ORR, resection rate, safety, exploratory biomarker analysis

*PFS, progression-free survival; defined as time from date of randomisation to date of first radiographic disease (per modified RECIST v1.0), or death within 60 days after the last evaluable tumour assessment or randomisation (whichever is later). Patients not meeting the criteria by the cut-off date were censored at the last evaluable tumour assessment date; OS, overall survival; ORR, objective response rate; mFOLFOX6, modified FOLFOX6

PEAK Trial Biomarker Analysis

PFS in Patients with WT KRAS exon 2 and WT RAS mCRC Treated with Panitumumab + mFOLFOX6

Original WT KRAS exon 2 (ITT set)

- Panitumumab + mFOLFOX6 (n=142)
  - Events: 90 (63)
  - Median (95% CI): 10.9 (9.4–13.0)
- Bevacizumab + mFOLFOX6 (n=143)
  - Events: 94 (66)
  - Median (95% CI): 10.1 (9.0–12.6)
  - HR*: 0.87 (95% CI: 0.65–1.17)
  - p=0.35

WT RAS (exons 2,3,4 of KRAS/NRAS)

- Panitumumab + mFOLFOX6 (n=88)
  - Events: 50 (57)
  - Median (95% CI): 13.0 (10.9–15.1)
- Bevacizumab + mFOLFOX6 (n=82)
  - Events: 60 (73)
  - Median (95% CI): 9.5 (9.0–12.7)
  - HR*: 0.65 (95% CI: 0.44–0.96)
  - p=0.03

PEAK Trial Expanded RAS Analysis

**KRAS**
- **EXON 1**: 12, 13 (N/A)
- **EXON 2**: 59, 61 (4%)
- **EXON 3**: 117, 146 (7%)

**NRAS**
- **EXON 1**: 12, 13 (5%)
- **EXON 2**: 59, 61 (6%)
- **EXON 3**: 117, 146 (0%)

**BRAF**
- **EXON 1**:
- **EXON 15**: 600 (6%)
- **EXON 16**:

Molecular pathways in CRC

- Wnt pathway
  - Fz
  - APC
  - B-catenin
  - TCF
  - MYC

- EGFR pathway

- HER2 pathway

- IGF pathway

- VEGF pathway

- RAF
  - RAS
  - PI3K
  - PTEN
  - AKT
  - mTOR
BRAF gene mutations

Exon 15, codon 600 (mutation V600E)
Molecular pathways in CRC
Predictive and Prognostic Analysis of \textit{PIK3CA} Mutation in Stage III Colon Cancer Intergroup Trial

Shuji Ogino, Xiaoyun Liao, Yu Imamura, Mai Yamauchi, Nadine J. McCleary, Kimmie Ng, Donna Niedzwiecki, Leonard B. Saltz, Robert J. Mayer, Renaud Whittom, Alexander Hantel, Al B. Benson III, Rex B. Mowat, Donna Spiegelman, Richard M. Goldberg, Monica M. Bertagnolli, Jeffrey A. Meyerhardt, Charles S. Fuchs; for the Alliance for Clinical Trials in Oncology

Manuscript received May 6, 2013; revised August 14, 2013; accepted August 15, 2013.

Background

Somatic mutations in \textit{PIK3CA} (phosphatidylinositol-4,5-bisphosphate 3-kinase [PI3K], catalytic subunit alpha gene) activate the PI3K-AKT signaling pathway and contribute to pathogenesis of various malignancies, including colorectal cancer.

Methods

We examined associations of \textit{PIK3CA} oncogene mutation with relapse, survival, and treatment efficacy in 627 stage III colon carcinoma case subjects within a randomized adjuvant chemotherapy trial (5-fluorouracil and leucovorin [FU/LV] vs irinotecan [CPT11], fluorouracil and leucovorin [IFL]; Cancer and Leukemia Group B 89803 [Alliance]). We detected \textit{PIK3CA} mutation in exons 9 and 20 by polymerase chain reaction and pyrosequencing. Cox proportional hazards model was used to assess prognostic and predictive role of \textit{PIK3CA} mutation, adjusting for clinical features and status of routine standard molecular pathology features, including \textit{KRAS} and \textit{BRAF} mutations and microsatellite instability (mismatch repair deficiency). All statistical tests were two-sided.

Results

Compared with \textit{PIK3CA} wild-type cases, overall status of \textit{PIK3CA} mutation positivity or the presence of \textit{PIK3CA} mutation in either exon 9 or 20 alone was not statistically significantly associated with recurrence-free, disease-free, or overall survival (log-rank $P > .70$; $P > .40$ in multivariable regression models). There was no statistically significant interaction between \textit{PIK3CA} and \textit{KRAS} (or \textit{BRAF}) mutation status in survival analysis ($P_{\text{interaction}} > .18$). \textit{PIK3CA} mutation status did not appear to predict better or worse response to IFL therapy compared with FU/LV therapy ($P_{\text{interaction}} > .16$).

Conclusions

Overall tumor \textit{PIK3CA} mutation status is not associated with stage III colon cancer prognosis. \textit{PIK3CA} mutation does not appear to serve as a predictive tumor molecular biomarker for response to irinotecan-based adjuvant chemotherapy.

J Natl Cancer Inst
# Molecular markers in CCR

<table>
<thead>
<tr>
<th>Study</th>
<th>RG</th>
<th>AC + QT vs. QT</th>
<th>SLP</th>
<th>AC + QT vs. QT</th>
<th>SG</th>
<th>AC + QT vs. QT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRYSAL33</td>
<td>57.3% vs. 39.7%</td>
<td>HR: 2.069; p &lt; 0.001</td>
<td>9.9 vs. 8.4 meses</td>
<td>HR: 0.696; p = 0.0012</td>
<td>23.5 vs. 20.0 meses</td>
<td>HR: 0.796; p = 0.0093</td>
</tr>
<tr>
<td>OPUS34</td>
<td>57.3% vs. 34.0%</td>
<td>HR: 2.551; p = 0.0027</td>
<td>8.3 vs. 7.2 meses</td>
<td>HR: 0.567; p = 0.0064</td>
<td>22.8 vs. 18.5 meses</td>
<td>HR: 0.855; p = 0.39</td>
</tr>
<tr>
<td>COIN37</td>
<td>64% vs. 57%</td>
<td>p = 0.049</td>
<td>8.6 vs. 8.6 meses</td>
<td>HR: 0.96; p = 0.60</td>
<td>17.9 vs. 17.0 meses</td>
<td>HR: 1.04; p = 0.67</td>
</tr>
<tr>
<td>PRIME40</td>
<td>57% vs. 48%</td>
<td>p = 0.018</td>
<td>10.0 vs. 8.6 meses</td>
<td>HR: 0.80; p = 0.009</td>
<td>23.9 vs. 19.7 meses</td>
<td>HR: 0.88; p = 0.072</td>
</tr>
</tbody>
</table>

**AC:** anticuerpo; **HR:** razón de riesgo; **QT:** quimioterapia; **RG:** respuesta global; **SG:** supervivencia global; **SLP:** supervivencia libre de progresión.
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- FAP; AFAP
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- Ashkenazi 11307K
- CHEK2 (HCC)
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- TGFBRI

= as yet undiscovered hereditary cancer variants

HT Lynch, 2013
Practical uses of CRC molecular pathology

• Therapy guidance
  – Targets
    • RAS
  – Microsatellite instability
    • BRAF

• Identification of patients at risk
  – Lynch Syndrome
  – MSI
Immunohistochemistry *hMLH1, hMSH2, hMSH6, hPMS-2*

**NORMAL:** Positive Nuclei: Functional protein. Gene integrity

**ABNORMAL:** Negative Nuclei: Non-functional protein. Gene alteration
IHC relation to gene alteration

<table>
<thead>
<tr>
<th>MLH1</th>
<th>PMS2</th>
<th>MSH2</th>
<th>MSH6</th>
<th>GEN ALTERADO</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>MLH1</td>
</tr>
<tr>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>PMS2</td>
</tr>
<tr>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>(-)</td>
<td>MSH2</td>
</tr>
<tr>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>MSH6</td>
</tr>
</tbody>
</table>

[Diagram showing hMutLα, hMutSα, MLH1, PMS2, MSH2, MSH6]
MLH1 negative protein / BRAF mutation

<table>
<thead>
<tr>
<th>PROTEÍNA MLH1</th>
<th>V600E BRAF</th>
<th>ALTERACIÓN GEN MLH1</th>
<th>CONCLUSIÓN</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)</td>
<td>MUTACIÓN</td>
<td>HIPERMETILACIÓN PROMOTOR</td>
<td>CCR ESPORÁDICO</td>
</tr>
<tr>
<td>(-)</td>
<td>WILD TYPE</td>
<td>MUTACIÓN</td>
<td>CCR HEREDITARIO</td>
</tr>
</tbody>
</table>

15-20 % sporadic CRC

Hypermethylation of *hMLH1* promoter area + BRAF mutation
Molecular alterations in CRC

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**Sporadic**
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- Lynch Syndrome

**Hereditary**
- AC-1 without MMR
  - (Familial CRC of syndrome “X”)
- \( TACSTD1 \) (EPCAM)
- Constitutional mosaic Epimutation (\( MLH1 \))

**CIMP +**
- MSI
- CIN

HT Lynch, 2013
CIMP - MSI

CpG island methylation
MLH1 promoter

Microsatellite instability
N
T
N + T

Bat-25
Bat-26

Loss of MLH1 expression

MLH1

CIMP - MSI

BRAF
BAX
IGFIIIR
MSH3
PTEN
Caspase 5
Bcl-10
APAF-1

T1
T2
A pie chart showing the distribution of responsive and non-responsive cases based on molecular aberrations. The responsive cases include:

- 15%: KRAS, BRAF, NRAS, PIK3CA wild type, no PTEN loss
- 5%: NRAS mutation
- 3%: BRAF + PIK3CA mutation

The non-responsive cases include:

- 32%: KRAS mutation
- 8%: KRAS + PIK3CA mutation
- 12%: PIK3CA mutation/PTEN loss
- 23%: Molecular aberration to be identified
Implications for Practice: Patients with stage II colon cancer have a low rate of recurrence after surgery. A modest benefit may be derived from chemotherapy after surgery, but this approach requires treating many patients who are already cured and who do not need treatment. A gene expression signature, ColoPrint, is able to distinguish patients with the highest risk of recurrence, who may derive greater benefit from further chemotherapy, and to reassure doctors and patients about the overall excellent prognosis of patients with a low-risk ColoPrint result. This study confirmed the performance of this test in a large cohort of patients across many different centers. This test is currently available commercially for patient testing.
Gene expression signatures

- **Oncotype Dx®** - 761 genes tested. 12 genes (7 prognostic value y 5 predictive value) validated as gene signatures. **FFPE material.** Prognostic value 5FU or FLOX: 3 risk categories (high, intermediate, low). No predictive value

- **ColoPrint®** - prognostic value for survival free of recurrence, DNA multigene Agilent de 44K. **Fresh material.** Gene signature 18 genes of high-low risk of recurrence in stage II patients

- **Colon Cancer DSA (ColDx®):** 634 genes. DNA microarray analysis **FFPE material.** Recurrence risk at 5 yrs.

**Recommendation:** Stage II MSI-CRC patients are not candidates for adjuvant therapy due to low risk of recurrence and low scientific evidence of therapy benefit (level IIa)
Stromal gene expression defines poor-prognosis subtypes in colorectal cancer

Alexandre Calon¹, Enza Lonardo¹, Antonio Berenguer-Llergo¹, Elisa Espinet¹,³, Xavier Hernando-Momblona¹, Mar Iglesias²,⁴, Marta Sevillano¹, Sergio Palomo-Ponce¹, Daniele V F Tauriello¹, Daniel Byrom¹, Carme Cortina¹, Clara Morral¹, Carles Barceló¹, Sebastien Tosi¹, Antoni Riera¹, Camille Stephan-Otto Attolini¹, David Rossell¹,⁶, Elena Sancho¹ & Eduard Batlle¹,⁵

Recent molecular classifications of colorectal cancer (CRC) based on global gene expression profiles have defined subtypes displaying resistance to therapy and poor prognosis. Upon evaluation of these classification systems, we discovered that their predictive power arises from genes expressed by stromal cells rather than epithelial tumor cells. Bioinformatic and immunohistochemical analyses identify stromal markers that associate robustly with disease relapse across the various classifications. Functional studies indicate that cancer-associated fibroblasts (CAFс) increase the frequency of tumor-initiating cells, an effect that is dramatically enhanced by transforming growth factor (TGF)-β signaling. Likewise, we find that all poor-prognosis CRC subtypes share a gene program induced by TGF-β in tumor stromal cells. Using patient-derived tumor organoids and xenografts, we show that the use of TGF-β signaling inhibitors to block the cross-talk between cancer cells and the microenvironment halts disease progression.
The consensus molecular subtypes of colorectal cancer


Received 6 March; accepted 6 September; published online 12 October 2015; doi:10.1038/nm.3967
Colorectal cancer (CRC) is a frequently lethal disease with heterogeneous outcomes and drug responses. To resolve inconsistencies among the reported gene expression–based CRC classifications and facilitate clinical translation, we formed an international consortium dedicated to large-scale data sharing and analytics across expert groups. We show marked interconnectivity between six independent classification systems coalescing into four consensus molecular subtypes (CMSs) with distinguishing features: CMS1 (microsatellite instability immune, 14%), hypermutated, microsatellite unstable and strong immune activation; CMS2 (canonical, 37%), epithelial, marked WNT and MYC signaling activation; CMS3 (metabolic, 13%), epithelial and evident metabolic dysregulation; and CMS4 (mesenchymal, 23%), prominent transforming growth factor–β activation, stromal invasion and angiogenesis. Samples with mixed features (13%) possibly represent a transition phenotype or intratumoral heterogeneity.

We consider the CMS groups the most robust classification system currently available for CRC—with clear biological interpretability—and the basis for future clinical stratification and subtype-based targeted interventions.
Comparison of published molecular subtyping platforms

Evaluation of 6 CRC subtyping algorithms, each developed independently using different gene expression data sets and analytical approaches

- 18 CRC data sets (n = 4,151 patients)
  - Public, The Cancer Genome Atlas (TCGA), private gene expression platforms (Affymetrix, Agilent and RNA-sequencing), sample collections (fresh-frozen and FFPE) and 1 clinical trial—were uniformly preprocessed and normalized from the raw formats to reduce technical variation
  - Application of an association network, nodes corresponded to the union of all group subtypes (n = 27), and weighted edges encoded the Jaccard similarity coefficients between nodes
  - Application of a Markov cluster (MCL) algorithm to this network to detect the presence of robust network substructures that would indicate recurring subtype patterns
Single expert teams subtyping approaches

- Six expert teams

- Different cohorts, platforms and subtyping methods

- Distinct colorectal cancer molecular subtypes

<table>
<thead>
<tr>
<th>Number of subtypes</th>
<th>Budinska et al.</th>
<th>Marisa et al.</th>
<th>Roepman et al.</th>
<th>De Sousa e Melo et al.</th>
<th>Sadanadam et al.</th>
<th>Schlicker et al.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 5</td>
<td>n = 6</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
</tbody>
</table>

Multiple expert teams subtyping approach

- Central repository: 18 data sets (n = 4,151)

- Normalized data

- Aggregated data (1 matrix)

- Independent expert team subtyping prediction: Predicted subtypes (n = 3,962)

- Network analysis for consensus subtype identification: Jaccard distance + MCL clustering x 1,000

- Core consensus samples (n = 3,104)

- Classifier construction and application in non-consensus samples

- Random forest algorithm

- Labeled samples (n = 3,443)

- Classifier

- Molecular and clinical subtype characterization: Annotated samples (n = 3,325)

- Sage Bionetworks
• Identification of 4 robust consensus molecular subtypes (CMSs) with significant interconnectivity (P < 0.001, hypergeometric test) among the six independent classification systems.

• ‘Mixed’ samples were not outliers and did not represent a fifth independent subtype, although the quality of gene expression data could have affected a small subset of samples.
<table>
<thead>
<tr>
<th>CMS1</th>
<th>CMS2</th>
<th>CMS3</th>
<th>CMS4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSI immune</td>
<td>Canonical</td>
<td>Metabolic</td>
<td>Mesenchymal</td>
</tr>
<tr>
<td>14%</td>
<td>37%</td>
<td>13%</td>
<td>23%</td>
</tr>
</tbody>
</table>

- **CMS1 (MSI immune)**
  - MSI, CIMP high, hypermutation
  - Immune infiltration and activation
  - Worse survival after relapse

- **CMS2 (Canonical)**
  - SCNA high
  - WNT and MYC activation

- **CMS3 (Metabolic)**
  - Mixed MSI status, SCNA low, CIMP low
  - Metabolic deregulation
  - Worse relapse-free and overall survival

- **CMS4 (Mesenchymal)**
  - SCNA high
  - Stromal infiltration, TGF-β activation, angiogenesis

**Diagram**

- Right colon: CMS1 (24%), CMS2 (31%), CMS3 (26%), CMS4 (19%)
- Left colon: CMS1 (27%), CMS2 (56%), CMS3 (10%), CMS4 (7%)
- Rectum: CMS1 (31%), CMS2 (3%), CMS3 (31%), CMS4 (15%)