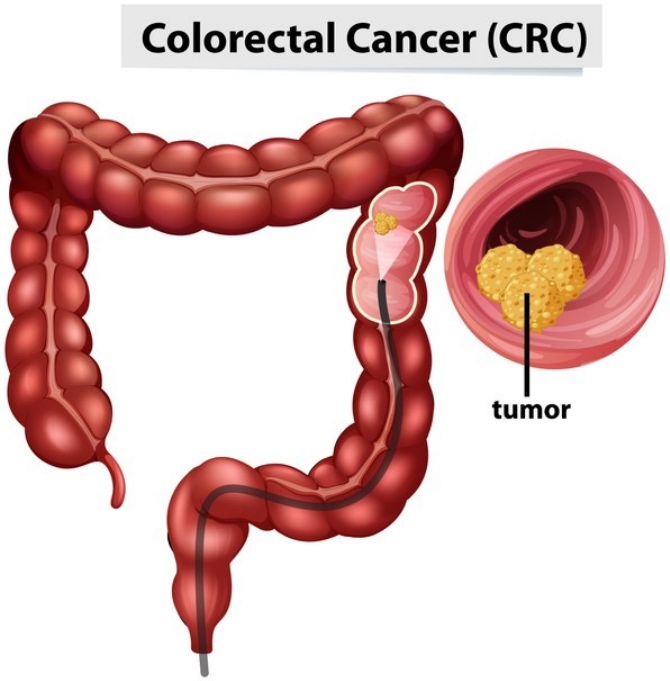
UNIVERSITÀ
DEGLI STUDI
DI TRIESTE

BIOMARKERS FOR CRC

Serena Bonin

Colorectal cancer CRC



https://www.freepik.com/free-vector/colorectal-cancer-crc-infographic-education_9956768.htm

CRC is the second and third leading cause of cancer death in men and women, respectively.

The vast majority of CRC develop **sporadically,** whereas <10% of cases result from a hereditary cancer syndrome.

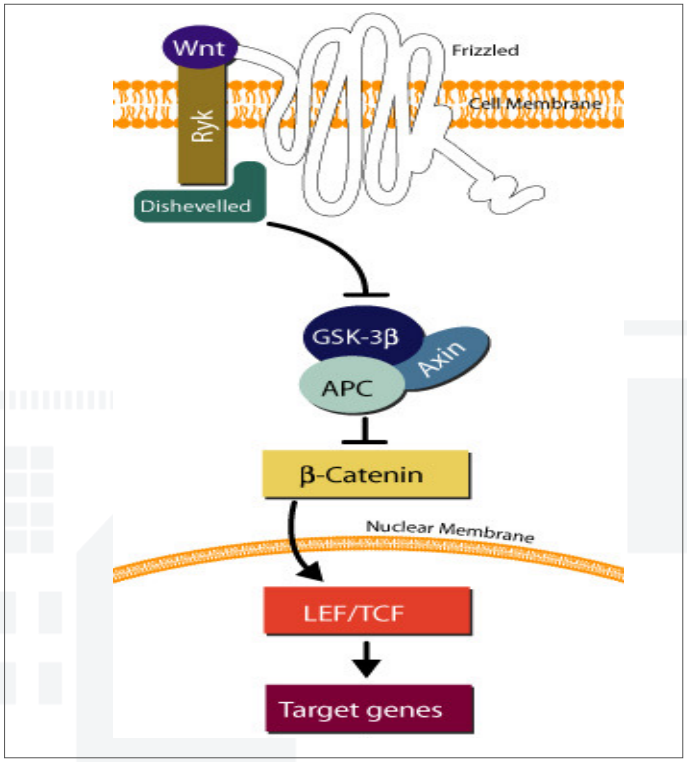
The majority of CRCs arise from **precursor lesions** such as adenoma, transforming to adenocarcinoma.

PROGRESSION FROM ADENOMA TO CARCINOMA

-It is generally accepted that **most colorectal carcinomas arise from adenomas**
-***APC*** was first identified as the gene mutated in familial adenomatous polyposis.[2] *APC* mutations are also present in the majority of sporadic colorectal cancers, where **mutations occur early during neoplastic development, and even in dysplastic aberrant crypt foci**.[2]

-**APC** is a component of the **Wingless pathway (WNT)**, **critical to embryonic development and intestinal epithelial renewal**.[2,3] *APC* mutations abrogate its role in binding **beta-catenin**, thereby **releasing beta-catenin from phosphorylation regulation by GSK3β** and allowing it to accumulate in the **nucleus**, where it is involved in **activating transcription of a number of other downstream targets, such as cyclin D and Myc.**[2]
APC is also involved in **cytoskeletal interactions** and has been **directly implicated in maintaining genome stability**.[2]

-Activating mutations in ***KRAS*** are present in about **40%** of colorectal carcinomas.[4]
KRAS mutations typically occur early, in aberrant crypt foci or small adenomas, and result in constitutive activation of the gene.
-Mutation and inactivation of the ***TP53*** gene occurs in about **50% to 70%** of carcinomas, often at the point of development of high-grade dysplasia.[5]



2. Fodde R: *Eur J Cancer* 2002; 38:867-871.
3. Moon RT, Bowerman B, Boutros M, Perrimon N:. *Science* 2002; 296:1644-1646.
4. Vogelstein B, Fearon ER, Hamilton SR, et al:. *N Engl J Med* 1988; 319:525-532
5. Leslie A, Carey FA, Pratt NR, Steele RJ: *Br J Surg* 2002; 89:845-860.

CLASSIFICATION OF GENETIC SYNDROMES THAT PREDISPOSE TO COLORECTAL CANCER

SYNDROME	INHERITED GENE DEFECT	RISK IN CARRIERS	ATTRIBUTABLE RISK
FAMILIAL ADENOMATOUS POLYPOSIS	APC	>90% BY 40 YR	<0.5%
ATTENUATED FAMILIAL ADENOMATOUS POLYPOSIS	APC	<90% BY 70 YR	<0.5%
JUVENILE POLYPOSIS SYNDROME	SMAD4, BMPRIA		<<0.5%
PEUTZ-JEGHERS SYNDROME	STK/LKB		<<0.5%
COWDEN SYNDROME	PTEN		<<0.5%
HEREDITARY NONPOLYPOSIS COLORECTAL CANCER	MLH1, MSH2, PMS2, MSH6	50%-90% BY 70 YR	2%-5%

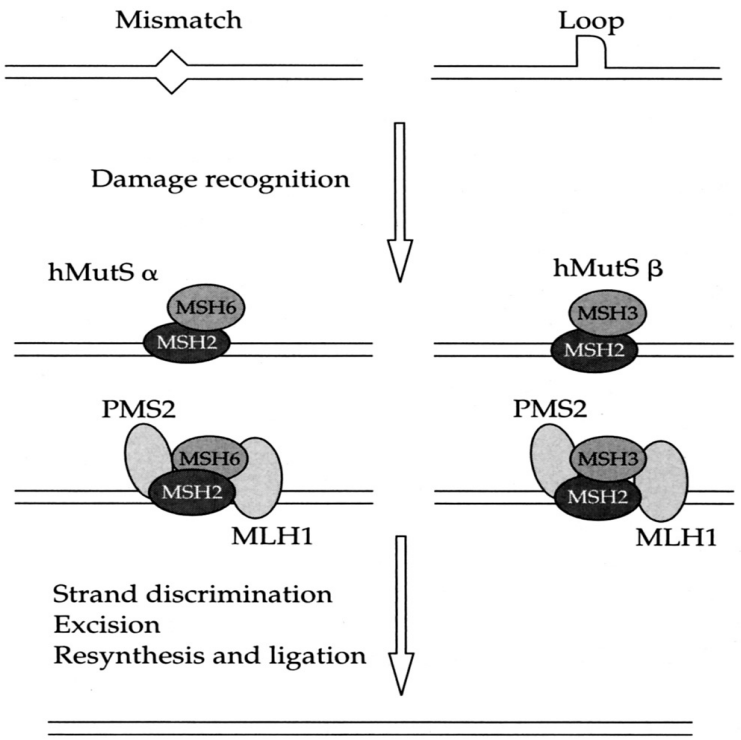
***BMPR1A** is a transmembrane serine/threonine kinases-ligands of these receptors are members of the TGF-β superfamily - represses WNT signaling to maintain stable stem cell populations and plays a role in cell differentiation.*

***DPC4 (deleted in PC locus 4) /SMAD4/MADH4**: is a tumor suppressor gene located at 18q21.1. It is part of the TGF-β signal transduction pathway (in the SMAD family are 9 members). SMAD-2 and -3 are directly phosphorylated by receptor kinases and are forming heteromeric complexes with SMAD-4. These complexes enter in the nucleus and bind to DNA for transcriptional activation of TGF-β responsive genes. SMAD-2/-4 and SMAD-3/-4 downregulate also c-myc and upregulate p21 and p15 (p21 inhibits CDK4/CD and CDK6/CD complexes).*

GENETIC BASIS OF HEREDITARY NONPOLYPOSIS COLORECTAL CANCER

GENE MUTATION
MLH1 (39%) MSH2 (38%)
MSH6 (11%)
PMS2 (7%)
UNKNOWN (5%)

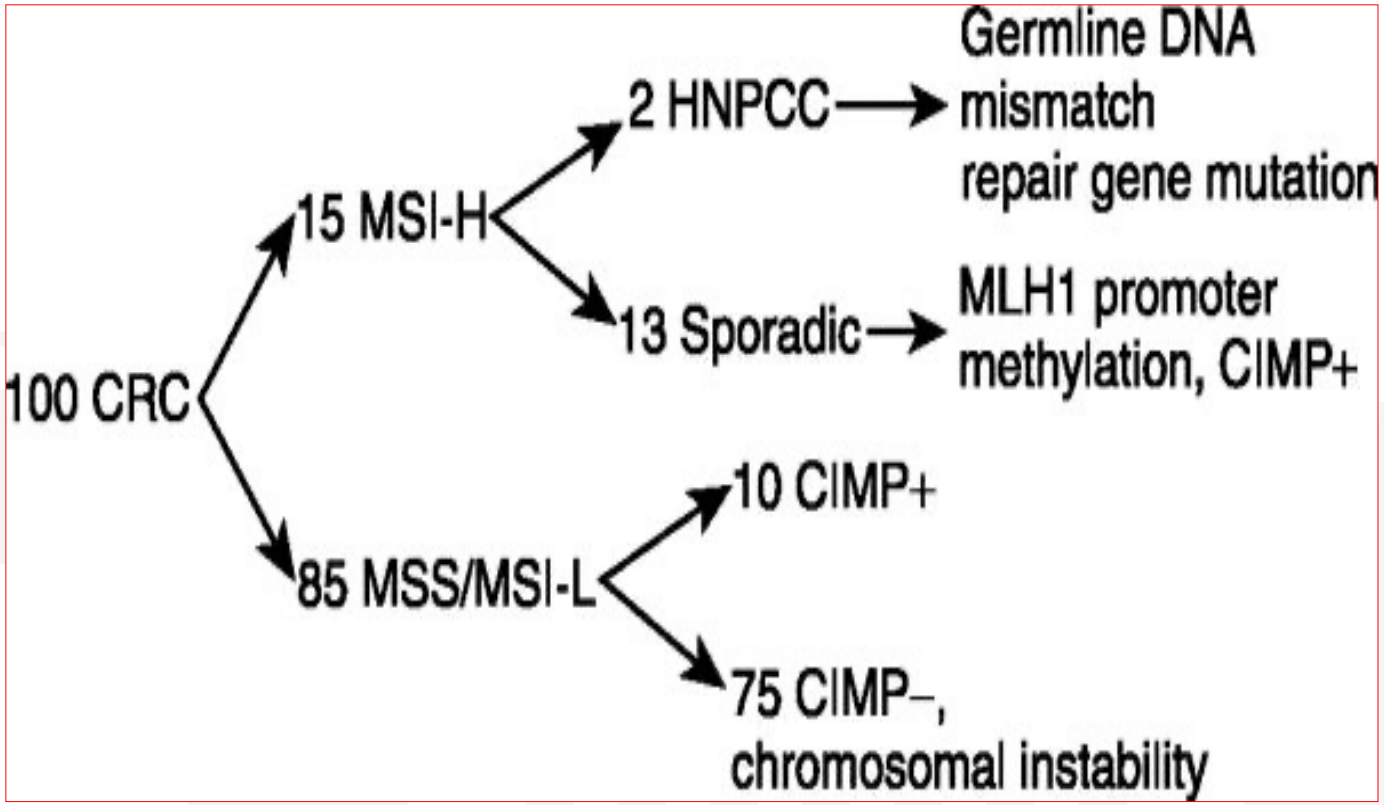
MISMATCH REPAIR



Mismatch repair is initiated by recognition and binding of MSH2-MSH6 or MSH2-MSH3, then MLH1 and PMS2 are recruited. Repair is completed by removal of the damage, resynthesis and ligation

From Woods MO, Williams P, Careen A, et al: A new variant database for mismatch repair genes associated with Lynch syndrome. Hum Mutat 28:669-673, 2007.

MOLECULAR CLASSIFICATION OF SPORADIC COLORECTAL CANCERS



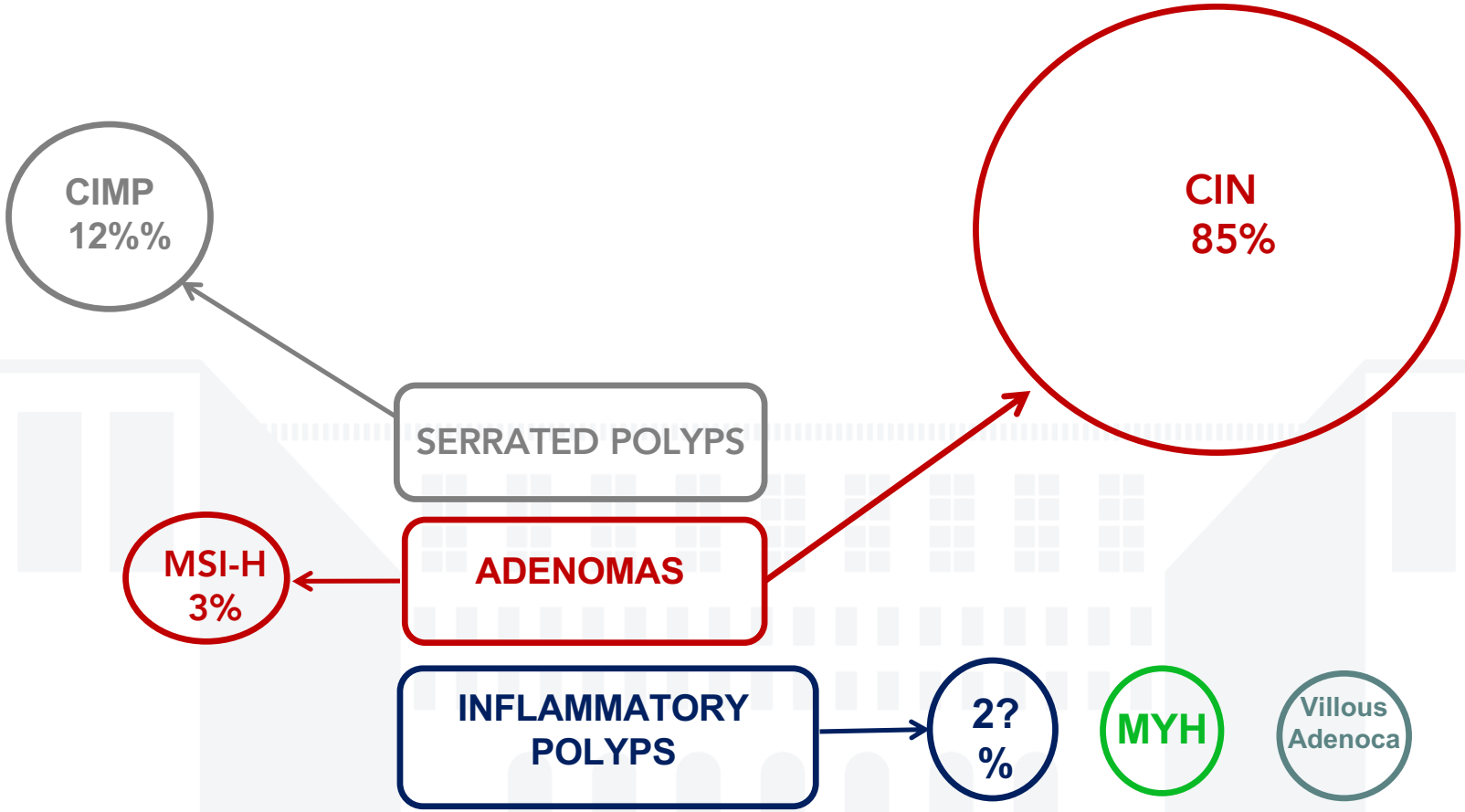
Three molecular carcinogenesis pathways have been identified;
(1)chromosomal instability (CIN),
(2) microsatellite instability (MSI),
(3) CpG island methylator phenotype (CIMP)

MOLECULAR PATHOLOGIC CLASSIFICATION OF COLORECTAL CANCER

GROUP NUMBER	CIMP STATUS	MLH1 STATUS	MICROSATELLITE INSTABILITY STATUS	CHROMOSOMAL STATUS	PRECURSOR	PROPORTION
1	CIMP HIGH	FULL METHYLATION	MSI-H	STABLE (DIPLOID)	SERRATED POLYP	12%
2	CIMP HIGH	PARTIAL METHYLATION	MSS/MSI-L	STABLE (DIPLOID)	SERRATED POLYP	8%
3	CIMP LOW	NO METHYLATION	MSS/MSI-L	UNSTABLE (ANEUPLOID)	ADENOMA/ SERRATED POLYP	20%
4	CIMP NEGATIVE	NO METHYLATION	MSS	UNSTABLE (ANEUPLOID)	ADENOMA	57%
5	CIMP NEGATIVE	GERMLINE MLH1 OR OTHER MUTATION	MSI-H	STABLE (DIPLOID)	ADENOMA	3%

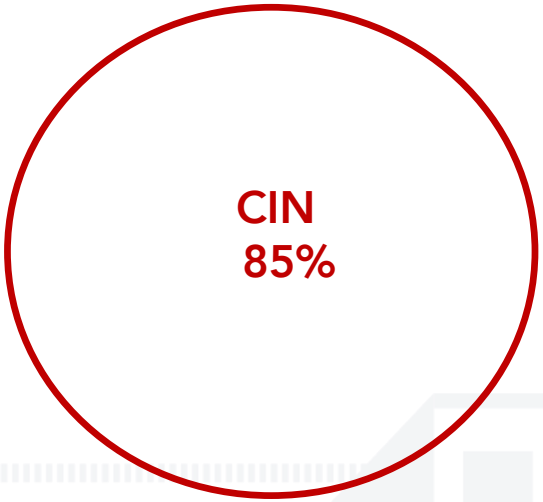
from Jass JR: Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 50:113-130, 2007.
CIMP, CpG island methylator phenotype; MSI-H, high-frequency microsatellite instability; MSI-L, low-frequency microsatellite instability; MSS, microsatellite stable.

HYPOTHETICAL CRC MOLECULAR CLASSIFICATION



CRC MOLECULAR CLASSIFICATION

CIN characterized by alteration of the number and structure of chromosomes, such as loss of chromosome 17p and 18q, leading to aneuploidy (an abnormal chromosome number), subkaryotypic amplification, chromosomal rearrangement, and loss of heterozygosity at tumor suppressor gene loci.
In addition, CIN tumors accumulate mutations in oncogenes and tumor suppressor genes including APC, TP53, KRAS, and BRAF

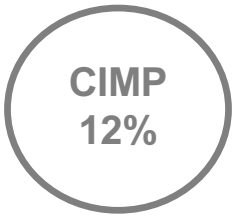


CRC MOLECULAR CLASSIFICATION

MSI-H
15%

MSI accounts for about 15% of CRCs, is characterized by generalized instability of short tandemly- repeated DNA sequences known as microsatellites. MSI may result from either mutation of one of mismatch repair (MMR) genes, MLH1, MSH2, MSH6, or PMS2, or silencing of the MLH1 promoter by hypermethylation.
Normally, when 2 strands of DNA replicate and nucleotide mismatch occur, these errors are corrected by a MMR enzyme.
Defects in this function result in a high frequency of replication errors because of the slippage of the DNA polymerase.
Lynch syndrome is the most common hereditary colon cancer syndrome.
Sporadic MSI tumors can occur because of methylation of CpG-rich promoter sequence of MLH1. These cancers tend to arise in proximal colon, tend to exhibit poor differentiation, mucinous cell type, and prominent lymphocytic infiltration. MSI tumors with hypermethylation account for 3/4 of hypermutated CRCs, whereas 1/4 had somatic MMR gene mutation and polymerase ε mutations.

HYPOTHETICAL CRC MOLECULAR CLASSIFICATION



CIMP pathway is characterized by widespread hypermethylation of numerous promoter CpG island loci and consequent inactivation of tumor suppressor genes. CIMP pathway accounts for 17% of CRC. Although CIN and MSI pathways are usually exclusive, the CIMP pathway overlaps substantially with the MSI pathway. In fact, sporadic MSI CRCs are almost exclusively associated with CIMP-associated methylation of the MLH1 promoter region. CIMP-positive tumors are shown to represent a distinct subset with high BRAF mutation. CIMP has a strong association with the serrated neoplasia. CIMP tumors tend to arise in the proximal colon, at an older age, and are more common in female individuals.

MSI TESTING IHC

4 major MMR proteins: MLH1, MSH2, PMS2, and MSH6

LIMITS: not all pathogenic mutations result in loss of expression and interpretation is somewhat subjective.

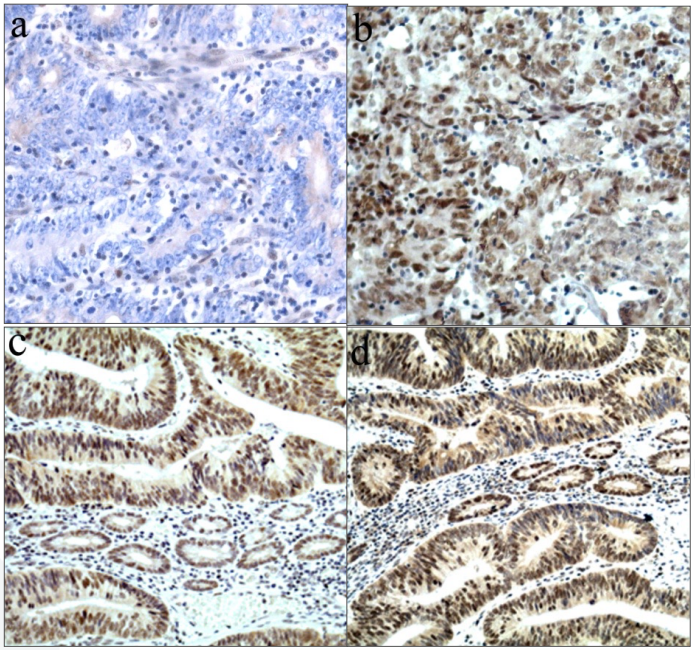
Interpretation of the test: all proteins are expressed in the nucleus → tumor microsatellite stable (MSS).

Loss of 1 or 2 protein expression → MMR deficiency, which highly correlates with MSI. As MLH1/PMS2 and MSH2/MSH6 form functional pairs and MLH1 and MSH2 are needed to stabilize the complex, when MLH1 or MSH2 are lost, PMS2 or MSH6 are also lost.

MLH1	PMS2	MSH2	MSH6	Interpretation	Action
+	+	+	+	MSS	No further action
–	–	+	+	MSI, MLH1 loss	MLH1 Promoter hyper methylation analysis , if no methylation MLH1 mutation analysis and genetic counselling
+	–	+	+	MSI, PMS2 loss	PMS2 mutation analysis and genetic counseling
+	+	–	–	MSI, MSH2 loss	MSH2 mutation analysis and genetic counseling
+	+	+	–	MSI, MSH6 loss	MSH6 mutation analysis and genetic counseling

MSI TESTING IHC

MLH1 MSH2



Immunohistochemical expression of MLH1 and MSH2 in colon adenocarcinomas. In a) and b) a tumor showing complete loss of MLH1 expression (a) and intact MSH2 expression (b). In c) and d) intact MLH1 c) and MSH2 expressions d) in the s ame tumor are reported. Nuclear immunostaining of normal epithelial cells (and lymphocytes) are used as internal positive controls. Pictures are at 20X magnification.

MSI TESTING IHC

MLH1	PMS2	MSH2	MSH6	Interpretation	Action
+	+	+	+	MSS	No further action
–	–	+	+	MSI, MLH1 loss	MLH1 Promoter hyper methylation analysis , if no methylation MLH1 mutation analysis and genetic counselling

Only a small percentage of MLH1 loss tumors are because of Lynch syndrome
The majority of them → silencing of MLH1 expression → promoter hypermethylation.
Hypermethylation of the MLH1 promoter → characteristic of CIMP tumors.

↓

BRAF p.V600E mutation.

↓

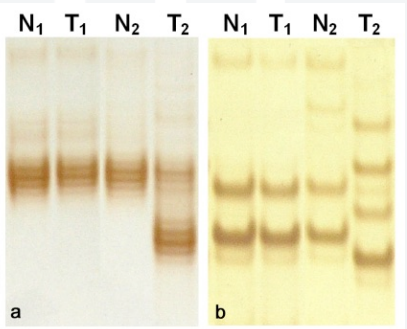
SPORADIC PATHOGENESIS

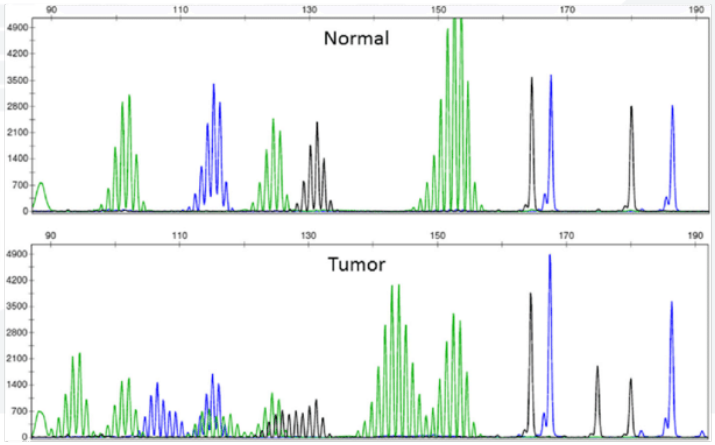
BRAF mutation → poor prognosis, especially in CIMP-low tumors

The absence of BRAF mutation does not exclude the sporadic etiology, but promoter methylation analysis is needed to exclude Lynch syndrome.
Loss of MSH2, MSH6, or PMS2 increases a probability for Lynch syndrome and genetic counselling and germline gene sequencing is recommended.
Germline mutations → nonsense or frameshift mutations → loss of function.

MSI TESTING PCR

MSI →alterations in the lengths of microsatellites, short tandem repeats
To standardize MSI analysis, → Bethesda panel, proposed in 1997 National Cancer Institute (NCI) → 5 microsatellite markers: 2 mononucleotide loci (BAT)-25 and BAT-26) and 3 dinucleotide loci (D2S123, D5S346, and D17S250)
Commercial assays → 5 nearly monomorphic mononucleotide microsatellite loci (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) → mononucleotide loci → more sensitive and specific than dinucleotide loci.
Interpretation of the test: MSI-H (high) tumor is defined → shift of the size in ≥ 2 out of 5 microsatellite loci, whereas a shift only at 1 locus → MSI-L (low).





COMPARISON OF ASSAY TECHNOLOGIES TO DETERMINE MISMATCH REPAIR GENE STATUS

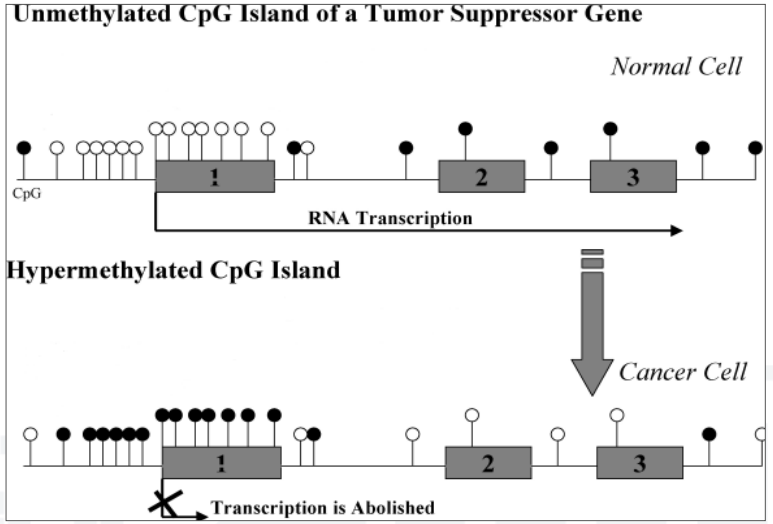
	MMR IHC	MSI PCR	AUTOMATED MSI	NGS
Description	MLH1, MSH2, MSH6, PMS2 protein expression determined by IHC	5 mononucleotide microsatellite loci, PCR, and fragment analysis	7 microsatellite loci, PCR, analyzed by high-resolution melting detection	Over 100 microsatellite loci, NGS analysis
Sensitivity **Specificity**	94% 100%	83%-98% 100%	No data No data	98% 100%
Pros	No need of molecular laboratory; work on low tumor cellularity samples; identify a defective gene	Require small amount of tumor; scalable; objective interpretation	Short hands-on time; fast turnaround time	Ability to analyze many more loci, reduce equivocal results
Cons	Some mutations do not result in expression loss; subjective interpretation	Need molecular lab; need normal tissue; labor intensive	Further validation study is needed; difficult to troubleshoot when failed	Expensive; need a special instrument and bioinformatics

METHODS TO ANALYZE CIMP

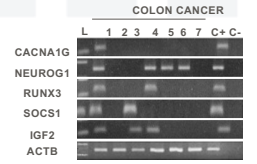
Marker	Orientation primers	GeneBank Number	Sequences	bp
CACNA1G	Forward Reverse	AC021491	tttttcgtttcgcgtttaggt ctcgaaacgacttcgccg	66
NEUROG1	Forward Reverse	AC005738	cgtgtagcttcgggtatttgta cgataattacgaacacactcc	87
RUNX3	Forward Reverse	AL023096	cgttcgatggtggacgtgt gacgaacaacgtcttattacaacg c	116
SOCS1	Forward Reverse	AC009121	gcgtcgagttcgtgggtattt ccgaaaccatcttcacgctaa	83
IGF2	Forward Reverse	AC132217	gagcggtttcggtgtcgtta ccaactcgatttaaaccacg	87
ACTB*	Forward Reverse	AT006483.3	tggtgatggaggaggaggtttagt aagt aaccaataaaacctactcctcc	133

* Widschwendter marker on Bisulfite treated DNA samples C→U; mC →C

At least 3 of 5 markers with a positive PCR amplification



Weisenberger panel



Representative samples of MSP for the methylated form of the markers shown in the table. Samples 1 and 4 were defined as CIMP+, all the others CIMP-. Samples 2 and 7 are negative for all the markers analyzed.

METHODS TO ANALYZE CIMP

BISULFITE TREATMENT

Genomic DNA amplification

mCGmCGTCTATGmCGAGGCmCGG

↓

mCGmCGTUTATGmCGAGGUmCGG

↓

CGCGTTTATGCGAGGTCGG

GCGCAAATACGCTCCAGCC

Bisulfite treatment

PCR amplification

When a CpG sequence is unmethylated

CGCGTCTATGCGAGGCCGG

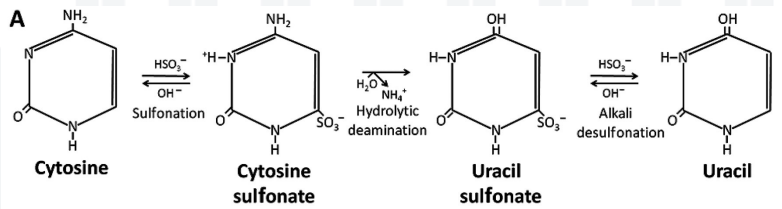
↓

UGUGTUTATGUGAGGUUGG

↓

TGTGTTTATGTGAGGTTGG

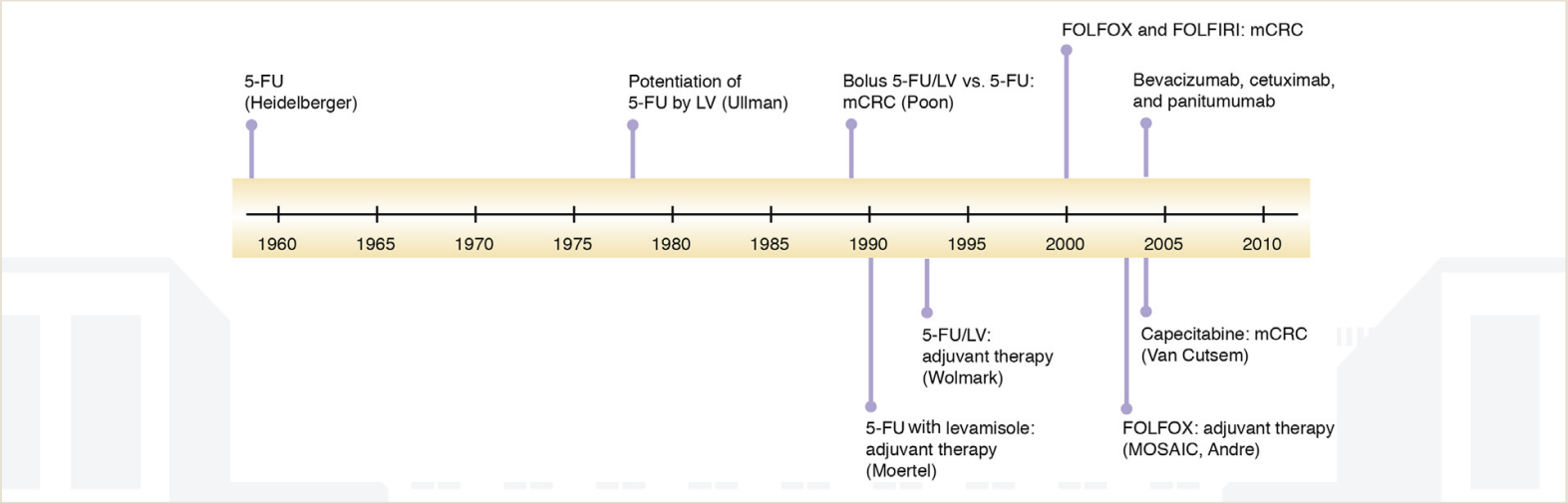
ACACAAATACACTCCAACC



B

	Original sequence	Sequence after bisulfite treatment
Unmethylated DNA	A-T-**C**-**G**-G-T-C-A-T-**C**-**G**-C-A-T	A-T-**U**-**G**-G-T-U-A-T-**U**-**G**-U-A-T
Methylated DNA	A-T-**C**-**G**-G-T-C-A-T-**C**-**G**-C-A-T	A-T-**C**-**G**-G-T-U-A-T-**C**-**G**-U-A-T

Figure 1 Landmark Advances in the Evolution of Systemic Chemotherapy for Patients With CRC



Abbreviations: 5-FU = 5-Fluorouracil; FOLFIRI = Infusional 5-FU/LV With Irinotecan; FOLFOX = 5-FU/LV With Oxaliplatin; LV = Leucovorin; mCRC = Metastatic Colorectal Cancer; MOSAIC = Multicenter International Study of Oxaliplatin/5-FU/Leucovorin in the Adjuvant Treatment of Colon Cancer.

B Gustavsson et al,Clinical Colorectal Cancer, 14, 1-10;2015

PREDICTIVE MARKERS IN CRC THERAPY

Clinical Predictive Goal	Molecular Genetic Marker
Benefit from chemotherapy in high-risk stage II disease	18q deletion Microsatellite stable
Response to fluorouracil	High thymidylate synthase expression Microsatellite stable
Response to irinotecan	High-frequency microsatellite instability
Response to cetuximab	Epithelial growth factor receptor amplification No KRAS mutation or BRAF mutation Amphiregulin expression present (EGFr ligand) Epiregulin expression present (EGFr ligand)
Response to preoperative chemotherapy and radiation therapy	TP53 intact
Response to oxaliplatin	None known
Response to bevacizumab (Avastin)	None known

TARGETS FOR BIOLOGICAL CANCER THERAPY

GATEKEEPERS
(proteins from tumor suppressors genes)

CARETAKERS
(proteins maintaining genome integrity)

} *MISSING FUNCTION IN CANCER CAN NOT BE REACTIVATED*

ONCOPROTEINS
(cellular growth and survival promoting) → *POSSIBLE DIRECT INHIBITION OR THAT OF DOWNSTREAM SIGNAL EFFECTORS*

BIOLOGICAL THERAPY

 # Humanized Monoclonal Antibodies
-the target is the receptor extracellular domain
-binding reversible only after receptor internalization

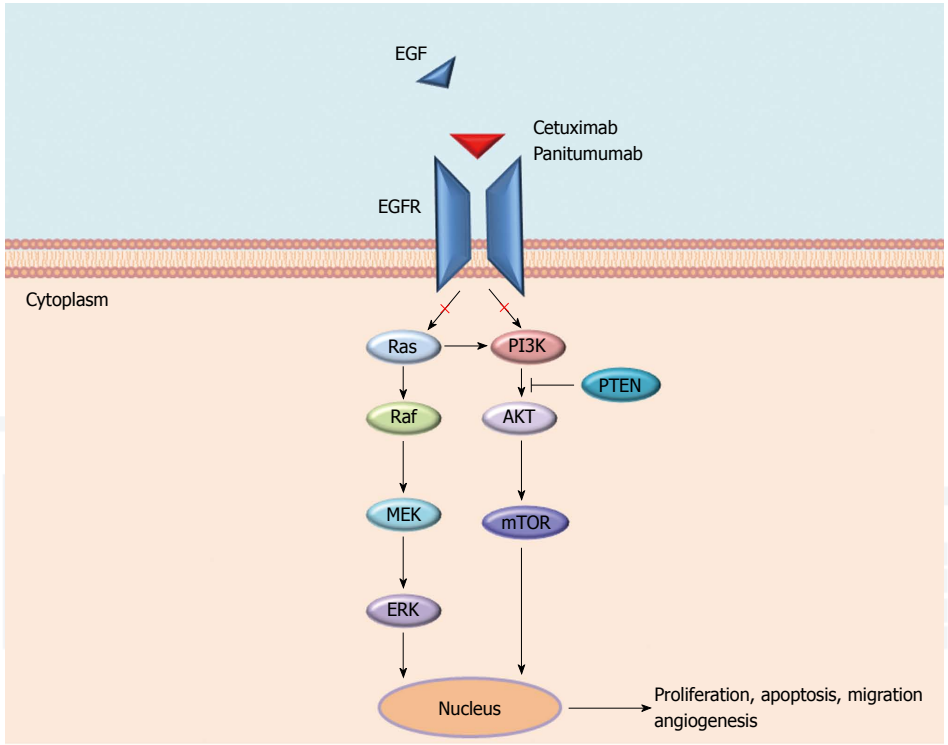
 # Small Organic Molecules
-the target is the tyrosine kinase domain
-binding can be reversible

BIOLOGICAL THERAPY IN CRC

Table 2 Three groups of FDA-approved targeted therapies for metastatic CRCs

Target	Examples	Mode of action	Comments
EGFR	Cetuximab	Monoclonal antibody to EGFR	First-line therapy: cetuximab + FOLFIRI or FOLFOX, overall survival was 23.5 months
	Panitumumab	Monoclonal antibody to EGFR	First-line therapy: panitumumab + FOLFOX, improved median overall survival of 26 months
VEGF	Bevacizumab	Monoclonal antibody to VEGFA	First-line therapy in combination with oxaliplatin-based therapy
	Aflibercept	Recombinant protein, decoy receptor for VEGFA, -B, and PlGF	Combination with FOLFIRI resulted in longer median overall survival and progression-free survival
Multikinase	Regorafenib	Tyrosine-kinase inhibitor of VEGFR1–3, TIE2	CORRECT trial

EGF-1



V Sforza et al, *World J Gastroenterol* 2016; 22(28): 6345-6361

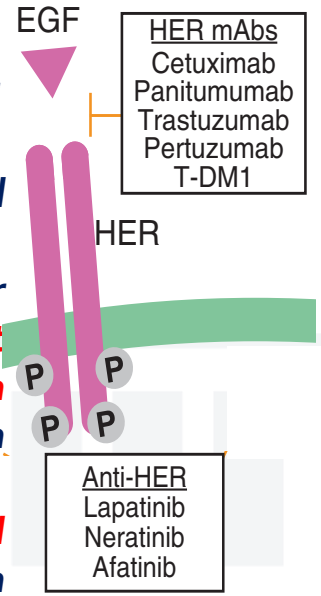
EGFR-targeted therapies

Cetuximab *(a recombinant chimeric IgG1 anti-EGFR mAb - 2005)*

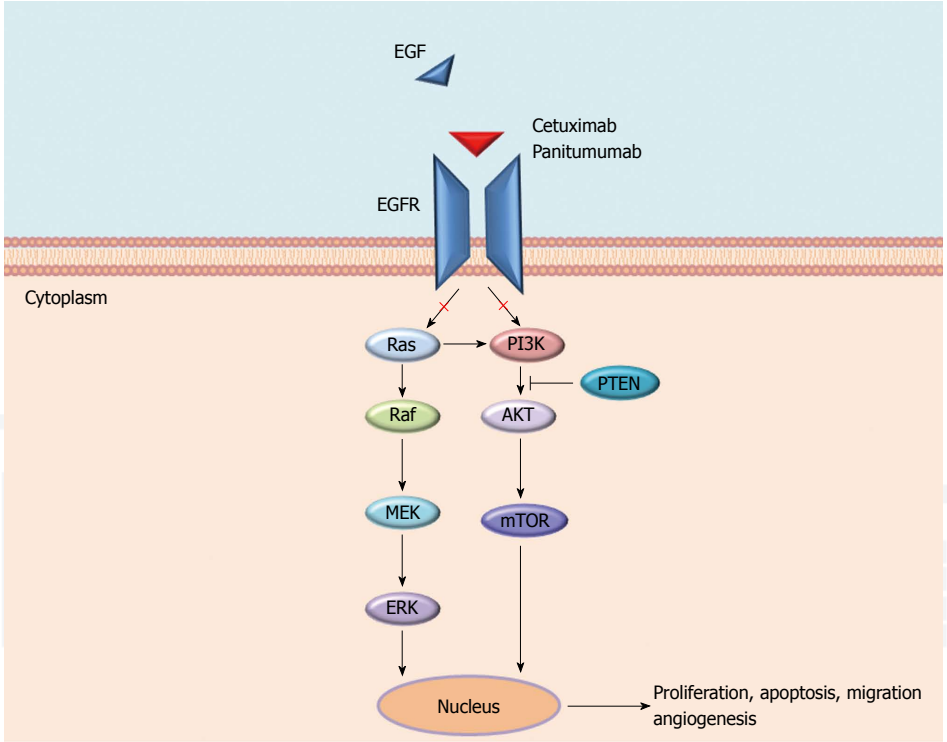
Panitumumab *(a fully human IgG2) approved with FOLFOX in 2006*

EGFR mutations at S492R -extracellular domain- are resistant to cetuximab, but sensitive to panitumumab. EGFR expression is not a useful marker, and no correlation with EGFR- gene amplification.

Amplification of EGFR or loss of PTEN indicate response to cetuximab, also with high expression of the EGFR ligands amphiregulin and epiregulin, and poor prognosis with high expression of TGFα.



EGF-1



V Sforza et al, *World J Gastroenterol* 2016; 22(28): 6345-6361

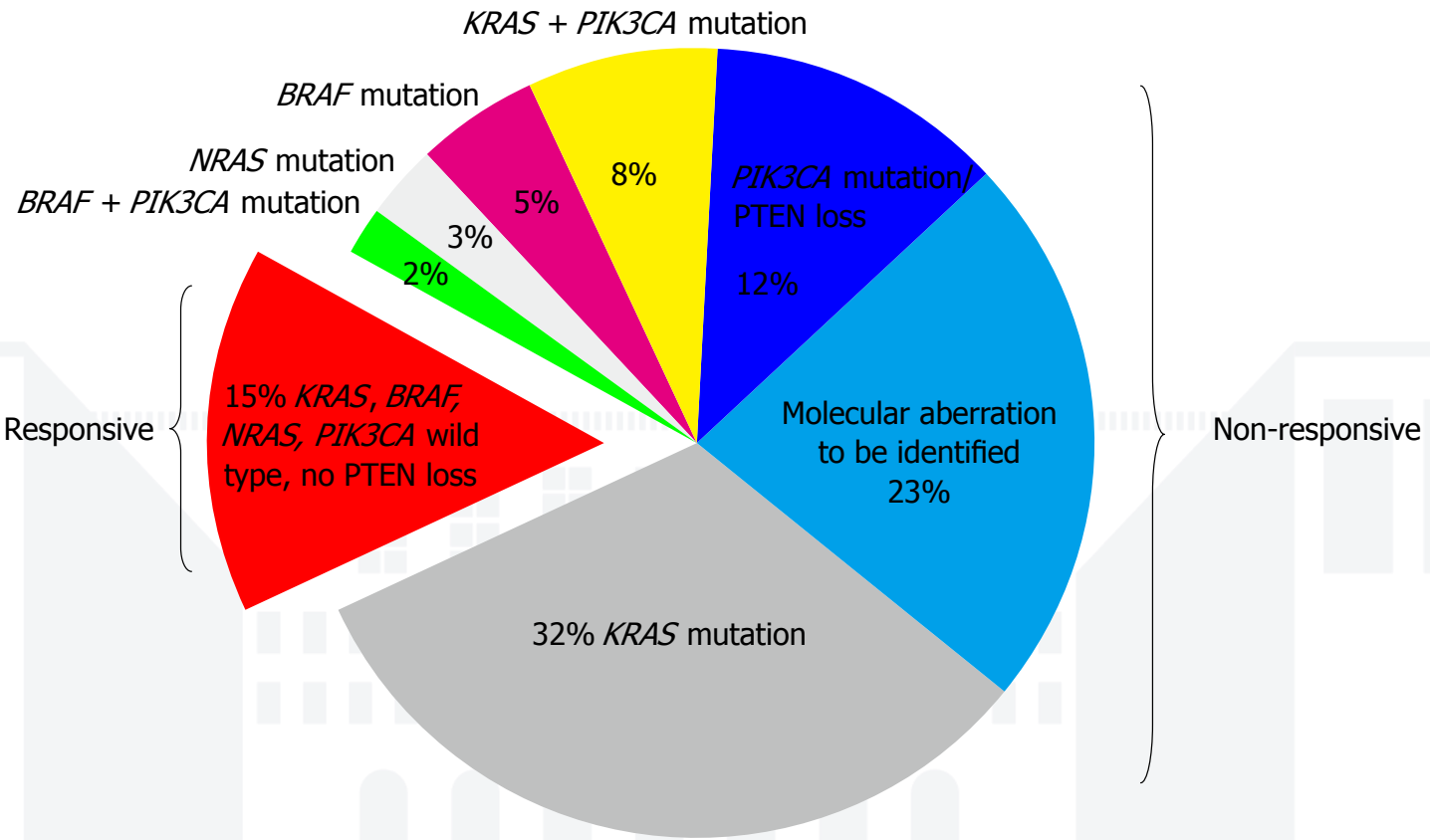
Activation mutations in the KRAS gene → 30% to 50% CRC.

Patients with KRAS mutation in codon 12 or 13 do not benefit from treatment with cetuximab or panitumumab.

In addition to codons 12 and 13 of KRAS, mutations in other codons of KRAS and in NRAS are found to render tumors resistant to anti-EGFR antibody therapy.

Mutational analysis should include KRAS and NRAS codons 12 and 13 of exon 2, 59, and 61 of exon 3, and 117 and 146 of exon 4 ("expanded" or "extended" RAS).

In addition, BRAF p.V600 [BRAF c.1799 (p.V600)] mutational analysis should be performed in CRC tissue in patients with colorectal carcinoma for prognostic stratification.



HY Luo, RH Xu , *World J Gastroenterol* 2014; 20(14): 3858-3874

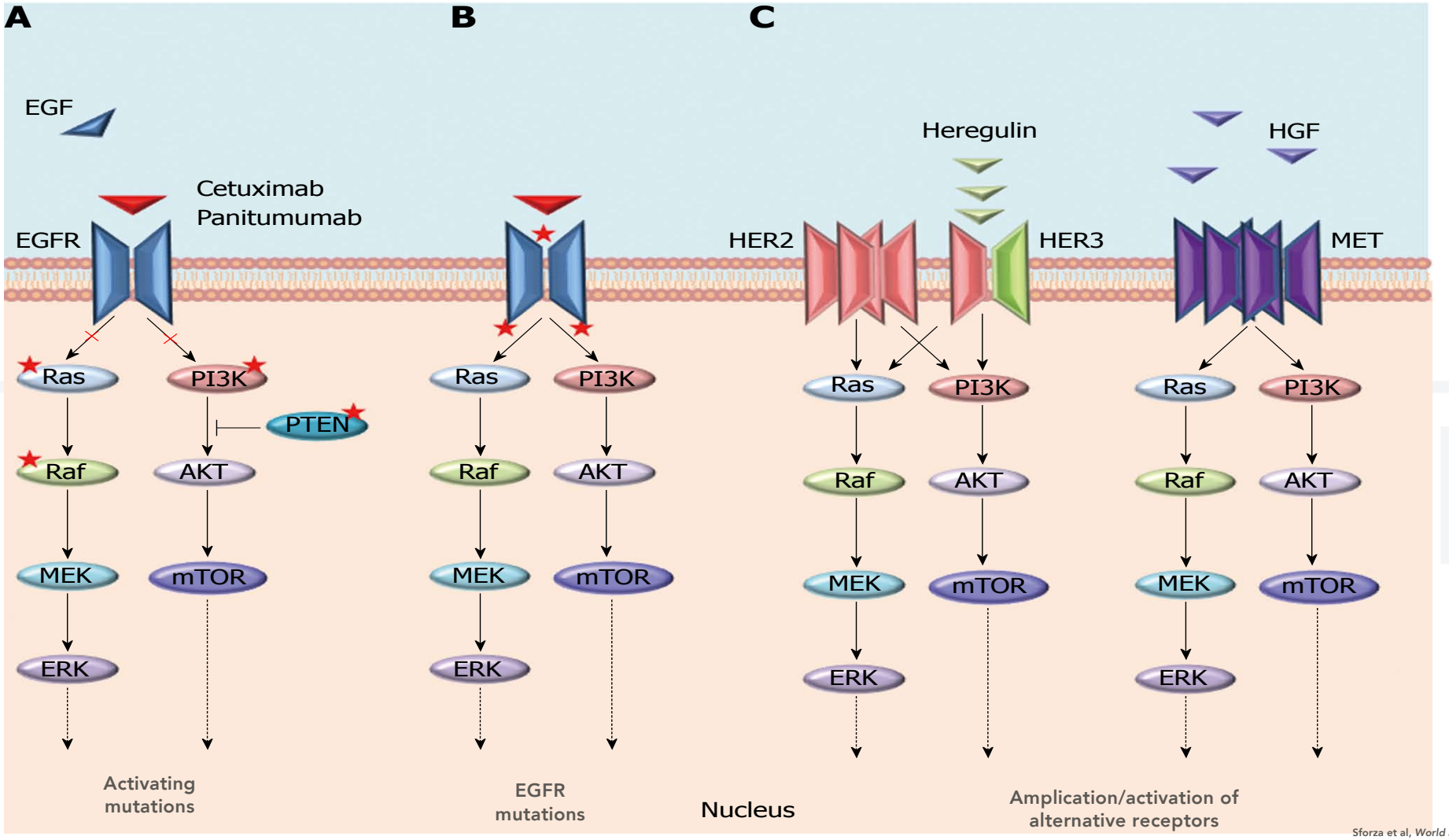
Gene symbol	Mutation status	Treatment outcome		*p* value*
		Responders (*n* = 30)	Non-responders (*n* = 23)	
BRAF	WT	30	17	0.004
	Mut	0	6	
KRAS	WT	30	18	0.012
	Mut	0	5	
NRAS	WT	29	20	0.305
	Mut	1	3	
BRAF/ KRAS/NRAS	WT	29	10	0.000
	Mut	1	13	
PIK3CA	WT	27	20	1.000
	Mut	3	3	
AKT1	WT	30	22	0.434
	Mut	0	1	
PTEN	WT	28	23	0.499
	Mut	2	0	
PIK3CA/ AKT1/ PTEN	WT	25	19	1.000
	Mut	5	4	
TP53	WT	11	9	1.000
	Mut	19	14	

*: Fisher exact *p*-value

Resistance :

Activation of effectors downstream of EGFR, such as mutant BRAF, PIK3CA, PTEN inactivation and PTEN loss, are associated with resistance. Approximately 25% of CRC patients with wild-type KRAS, BRAF, PIK3CA, and PTEN do not respond to cetuximab.

Other mechanisms include amplification of MET, overexpression of IGF1R, overexpression of EGFR ligands and receptors, such as ErbB2 and amphiregulin, modulation of EGFR by Src-family kinases, transactivation of alternative pathways that bypass the EGFR pathway, such as MET and IGFR, ubiquitination, expression of EGFR variant III, and induction of EGFR translocation.



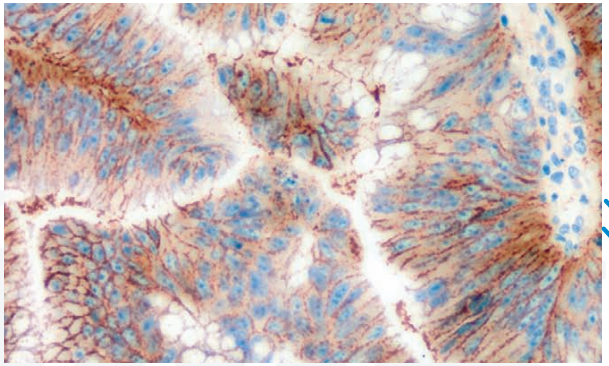
Sforza et al, *World J Gastroenterol* 2016; 22: 6345-6361

FDA approved test

GENE NAME	METHOD	PATHOLOGY	DRUG
FOR CRC: MSI TEST, KRAS (EXON 2,3,4), NRAS (EXON 2,3,4	NGS ONCOLOGY PANEL	COLORECTAL CANCER, FFPE	ERBITUX (CETUXIMAB) VECTIBIX (PANITUMUMAB)
KRAS (EXON 2,3,4), NRAS (EXON 2,3,4)	NGS	COLORECTAL CANCER, FFPE	VECTIBIX (PANITUMUMAB)
KRAS -SEVEN SOMATIC MUTATIONS IN CODONS 12 AND 13 (2 PROVIDERS)	FAST REAL-TIME PCR	COLORECTAL CANCER, FFPE	ERBITUX (CETUXIMAB) VECTIBIX (PANITUMUMAB)
EGFR	IHC		ERBITUX (CETUXIMAB) VECTIBIX (PANITUMUMAB)
BRAFV600E	FAST REAL-TIME PCR	COLORECTAL CANCER, FFPE	BRAFTOVI (ENCORAFENIB) –COMBINATION WITH ERBITUX (CETUXIMAB))

FDA approved test

GENE NAME	METHOD	PATHOLOGY	DRUG
EGFR	IHC		ERBITUX (CETUXIMAB) VECTIBIX (PANITUMUMAB)

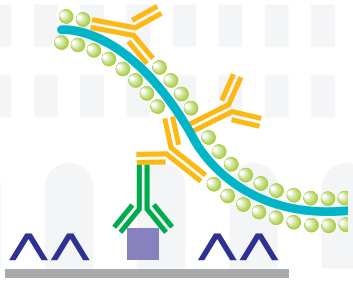


Step 1
Application of Peroxidase Block.
Incubate 5 minutes.

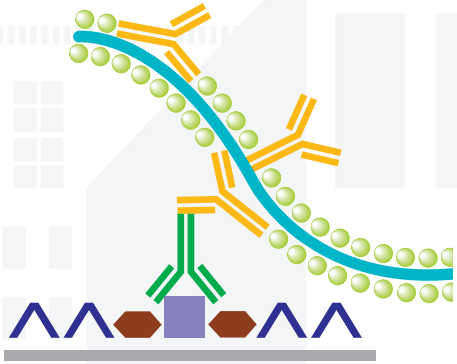
Step 2
Application of Proteinase K.
Incubate 5 minutes.

Step 3
Application of Primary Antibody.
Incubate 30 minutes.

Step 4
Application of Labeled Polymer,
HRP. Incubate 30 minutes.



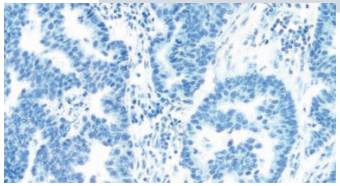
Step 5
Application of Substrate-Chromogen.
Incubate 10 minutes.



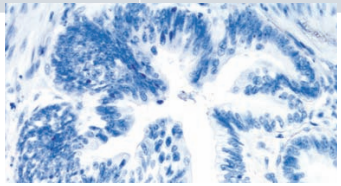
FDA approved test

GENE NAME	METHOD	PATHOLOGY	DRUG
EGFR	IHC		ERBITUX (CETUXIMAB) VECTIBIX (PANITUMUMAB)

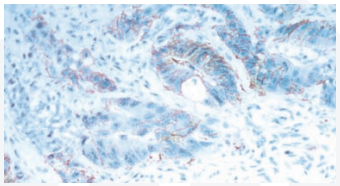
NEGATIVE RESULTS



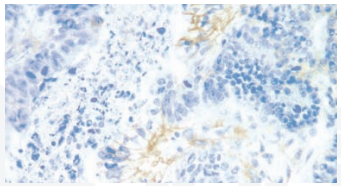
Colorectal adenocarcinoma, no staining, **0 staining intensity**; 10x magnification.



Colorectal adenocarcinoma, no staining, **0 staining intensity**; 20x magnification.

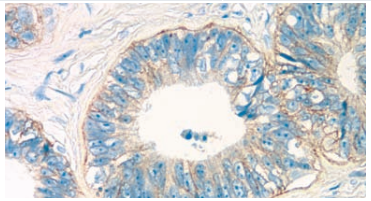


Colorectal adenocarcinoma, membrane staining, **1+ staining intensity**; 20x magnification.

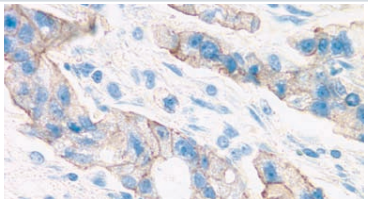


Colorectal adenocarcinoma, membrane staining, **1+ staining intensity**; 40x magnification.

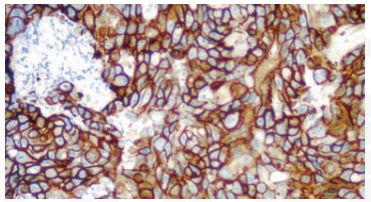
POSITIVE RESULTS



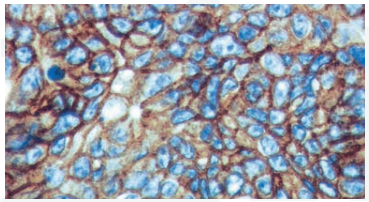
Colorectal adenocarcinoma, membrane staining, **2+ staining intensity**; 20x magnification.



Colorectal adenocarcinoma, membrane staining, **2+ staining intensity**; 40x magnification.



Colorectal adenocarcinoma, membrane staining, **3+ staining intensity**; 20x magnification.



Colorectal adenocarcinoma, membrane staining, **3+ staining intensity**; 40x magnification.

EGFR-Negative Tumor	Absence of membrane staining above background in all tumor cells.
EGFR-Positive Tumor	EGFR-positive staining is defined as **any** IHC staining of tumor cell **membranes** above background level; whether it is complete or incomplete circumferential staining.

Staining Intensity	Percent of Tumor Cells Staining
1+, 2+, or 3+	>0%

FDA approved test

GENE NAME	METHOD	PATHOLOGY	DRUG
EGFR	IHC		ERBITUX (CETUXIMAB) VECTIBIX (PANITUMUMAB)

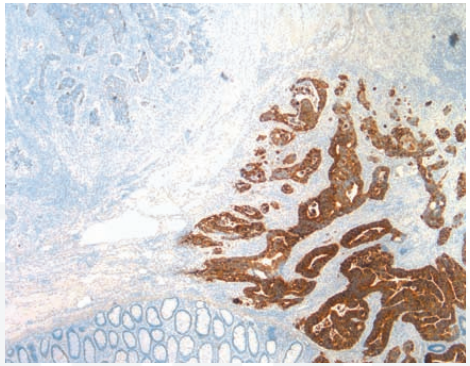


Figure 8
Colorectal adenocarcinoma, example of heterogeneous **positive** staining;
10x magnification.

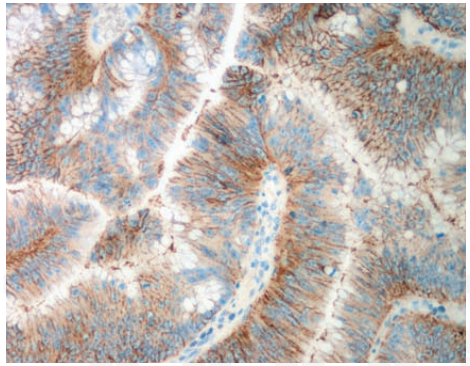


Figure 10
Colorectal adenocarcinoma, example of homogeneous **positive** staining;
20x magnification.

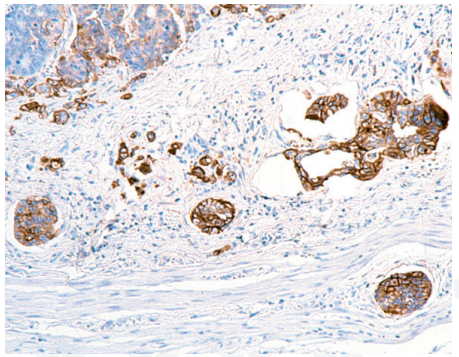


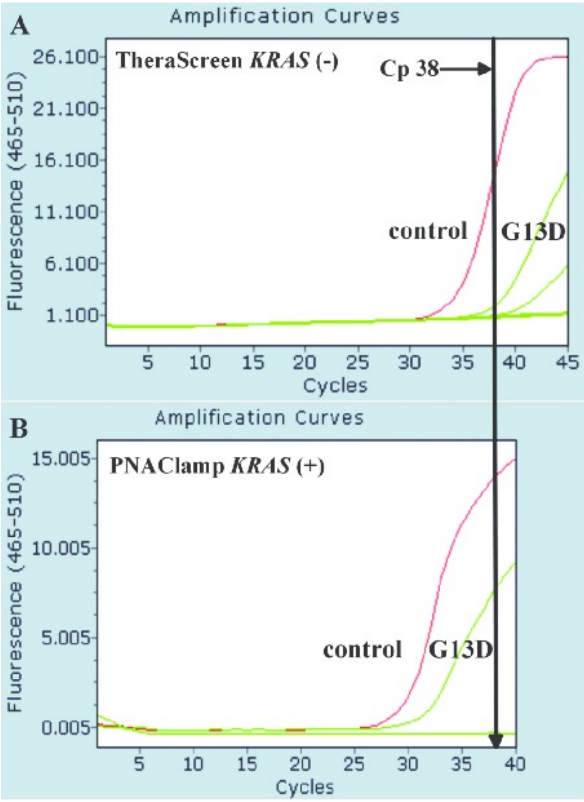
Figure 9
Colorectal adenocarcinoma, example of leading edge heterogenous **positive** staining;
10x magnification.

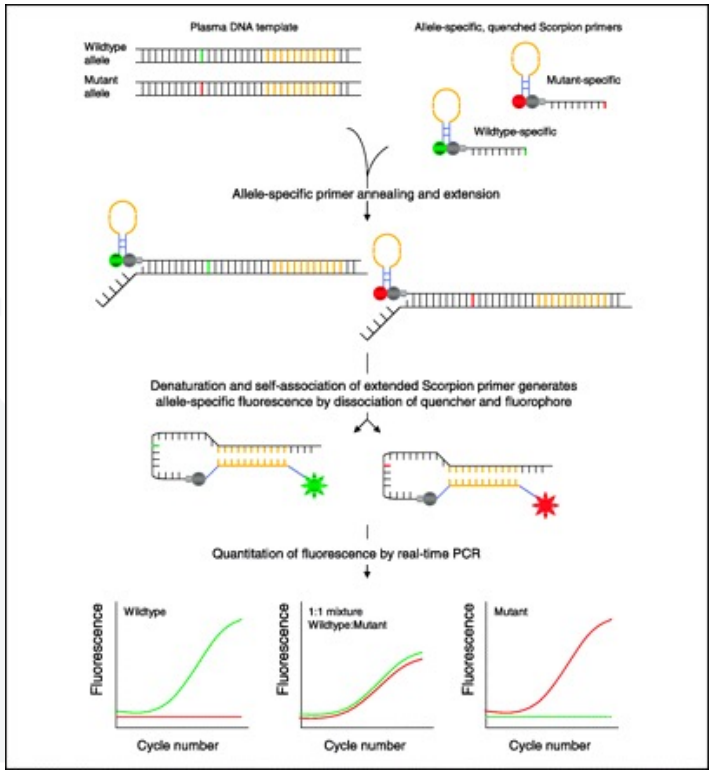
EGFR-Negative Tumor	Absence of membrane staining above background in all tumor cells.
EGFR-Positive Tumor	EGFR-positive staining is defined as **any** IHC staining of tumor cell **membranes** above background level; whether it is complete or incomplete circumferential staining.

Staining Intensity	**Percent of Tumor Cells Staining**
1+, 2+, or 3+	>0%

FDA approved test

GENE NAME	METHOD	PATHOLOGY	DRUG
KRAS -SEVEN SOMATIC MUTATIONS IN CODONS 12 AND 13 (2 PROVIDERS)	FAST REAL-TIME PCR	COLORECTAL CANCER, FFPE	ERBITUX (CETUXIMAB) VECTIBIX (PANITUMUMAB)





- ✓ DNA sample assessment
- ✓ Detection of KRAS mutations
- ✓ 8 separate PCR amplifications: 7 mutation-specific reactions in codons 12 and 13 of exon 2 of the KRAS oncogene, and a wild-type control in exon 4
- ✓ ARMS analysis
- ✓ Detection of amplification is performed using Scorpions.

ΔCT = [mutation assay CT value] – [control assay CT value]

Based on predetermined analytical CT and ΔCT values, the instrument software qualitatively determines the mutation status of the DNA samples and reports which samples contain which mutation.

✓

FDA approved test

GENE NAME	METHOD	PATHOLOGY	DRUG
KRAS -SEVEN SOMATIC MUTATIONS IN CODONS 12 AND 13 (2 PROVIDERS)	FAST REAL-TIME PCR	COLORECTAL CANCER, FFPE	ERBITUX (CETUXIMAB) VECTIBIX (PANITUMUMAB)

Table 10. Established cut-off values for each mutation assay

	Mutant assay (ΔC_T)						
	12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
Cut-off (ΔC_T) ≤	8.0	6.6	8.0	8.0	8.0	7.5	7.5

For sections that are ≤20% tumor content by area, macrodissect one or more sections. Discard the non-tumor tissue.

Sample control reaction range: 21,92-32,00. It means that Sample control reaction CT>32.00, will display "Invalid": Quantity of DNA is not sufficient for mutation analysis. Similarly, sample control reaction CT<21.92, will display "Invalid": DNA concentration is too high for mutation analysis .

Further controls: Positve control, NTC

Samples are classed as mutation positive: if they give a ΔCT ≤ to the cutoff ΔCT value for that assay.

Above this value, the sample may either contain less than the percentage of mutation able to be detected by the assay (beyond the limit of the assays), or the sample is mutation negative which would be reported as "No Mutation Detected". No amplification in mutation reactions will be scored as "No Mutation Detected". ΔCT values calculated from background amplification are expected to be greater than the cutoff ΔCT values and the sample will be classed as "No Mutation Detected".