

# Molecular diagnostics of solid tumors

Wade S. Samowitz, M.D.  
University of Utah and ARUP



## **THE TEN COMMANDMENTS**

- I Thou shalt have no other gods before me.
- II Thou shalt not make unto thee any graven image, or any likeness of anything that is in heaven above, in earth beneath, or in the water under the earth.
- III Thou shalt not take the name of the Lord thy God in vain, for the Lord will not hold him guiltless that taketh his name in vain.
- IV Remember the Sabbath day, to keep it holy.
- V Honour thy father and thy mother; that thy days may be long upon the land.
- VI Thou shalt not kill.
- VII Thou shalt not commit adultery.
- VIII Thou shalt not steal.
- IX Thou shalt not bear false witness against thy neighbour.
- X Thou shalt not covet thy neighbour's house, wife, manservant, maidservant, ox, ass, nor anything that is thy neighbour's.

Exodus 20:7-17

# Disclosures

- Potential royalties in the future related to the Ventana BRAF V600E antibody



# Case 1

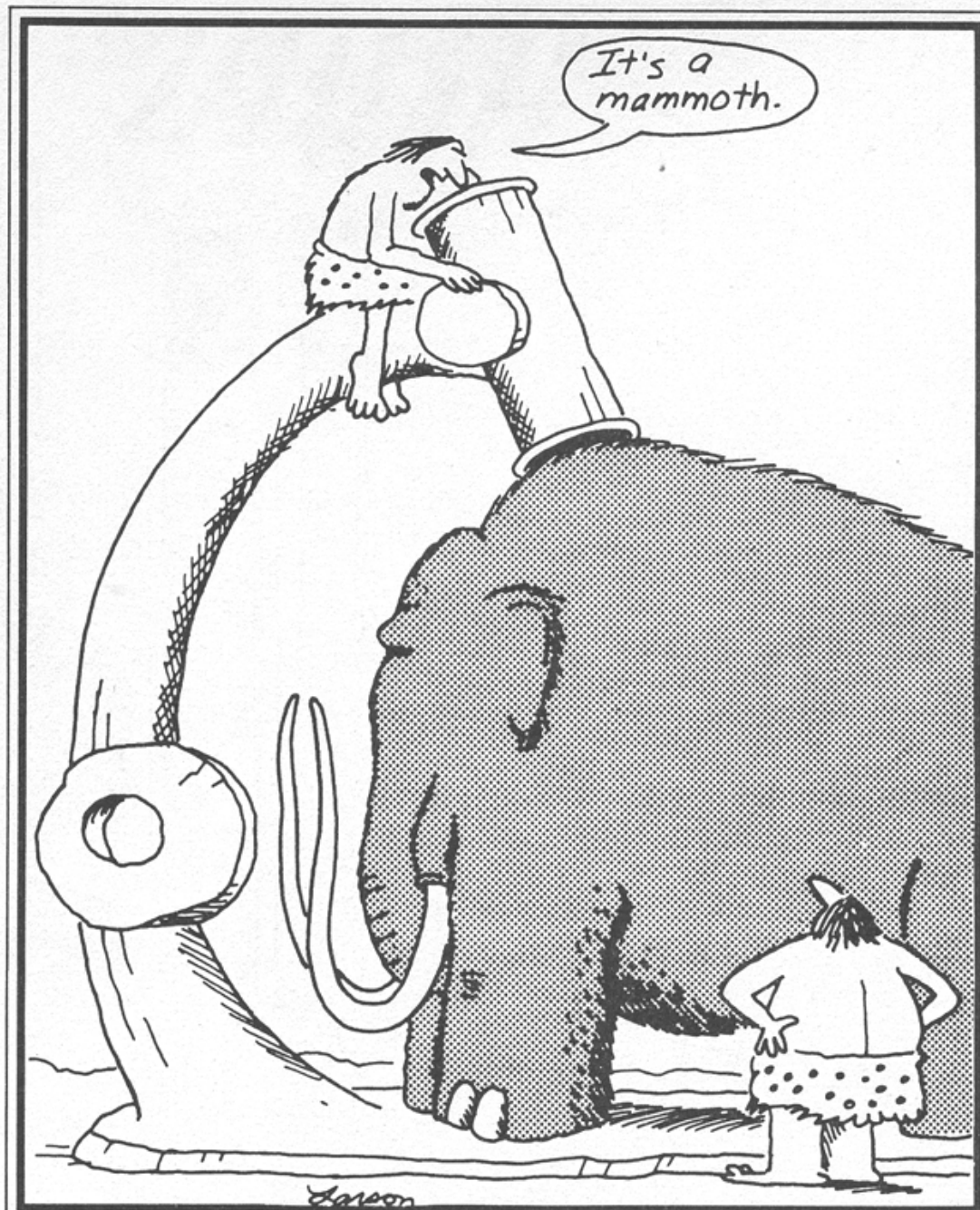
- 70 year old man with metastatic rectal cancer
- Oncologist wishes to treat with cetuximab, orders KRAS testing
- Patient received preoperative chemoradiation; initial slides of resection show pools of mucin with rare malignant glands
- The pre-treatment biopsy is requested

# Mutation detection in fixed tissue: General Considerations

- Solid tumors are different than germline DNA (or even most hematolymphoid samples)
  - Consist of heterogeneous cell types
  - Requires some form of microdissection
  - Need AP/CP coordination
- Garbage in, garbage out
  - Choose best tumor block (highest concentration of tumor)



Slide of a colon cancer with a circled area of colon cancer which will be microdissected



Early microscope

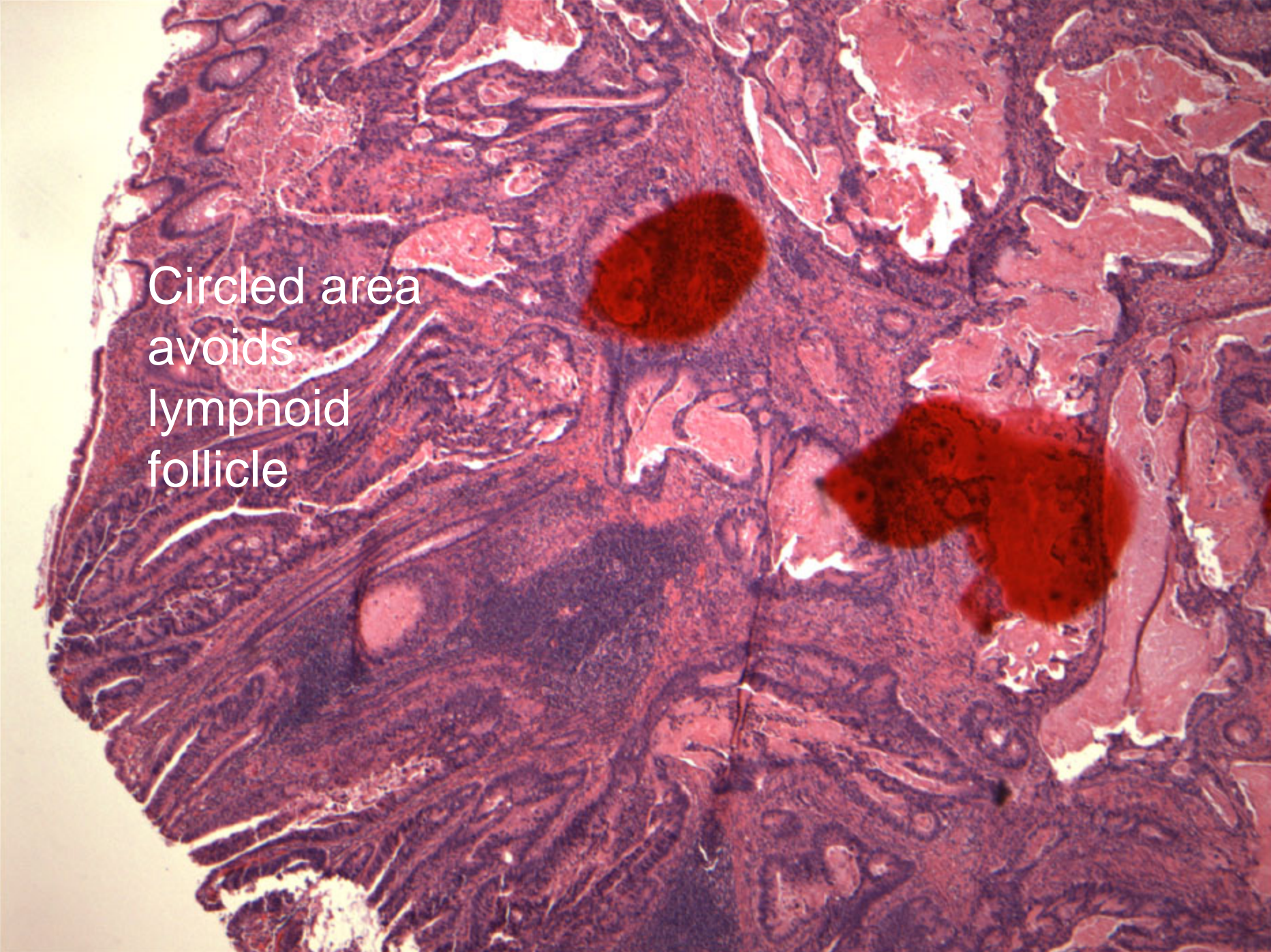


This is a high-magnification histological image of endometrial tissue stained with hematoxylin and eosin (H&E). The image shows a dense population of glandular structures, which are the uterine glands, embedded within a cellular stroma. The glands vary in size and shape, some appearing as simple tubular structures while others are more complex or branched. The stroma is composed of spindle-shaped cells, likely fibroblasts, and contains numerous small, dark-staining nuclei. The overall appearance is that of a hyperplastic or neoplastic endometrial tissue. A small, white, circular artifact is visible in the upper left quadrant of the image.

Higher power  
of circled area

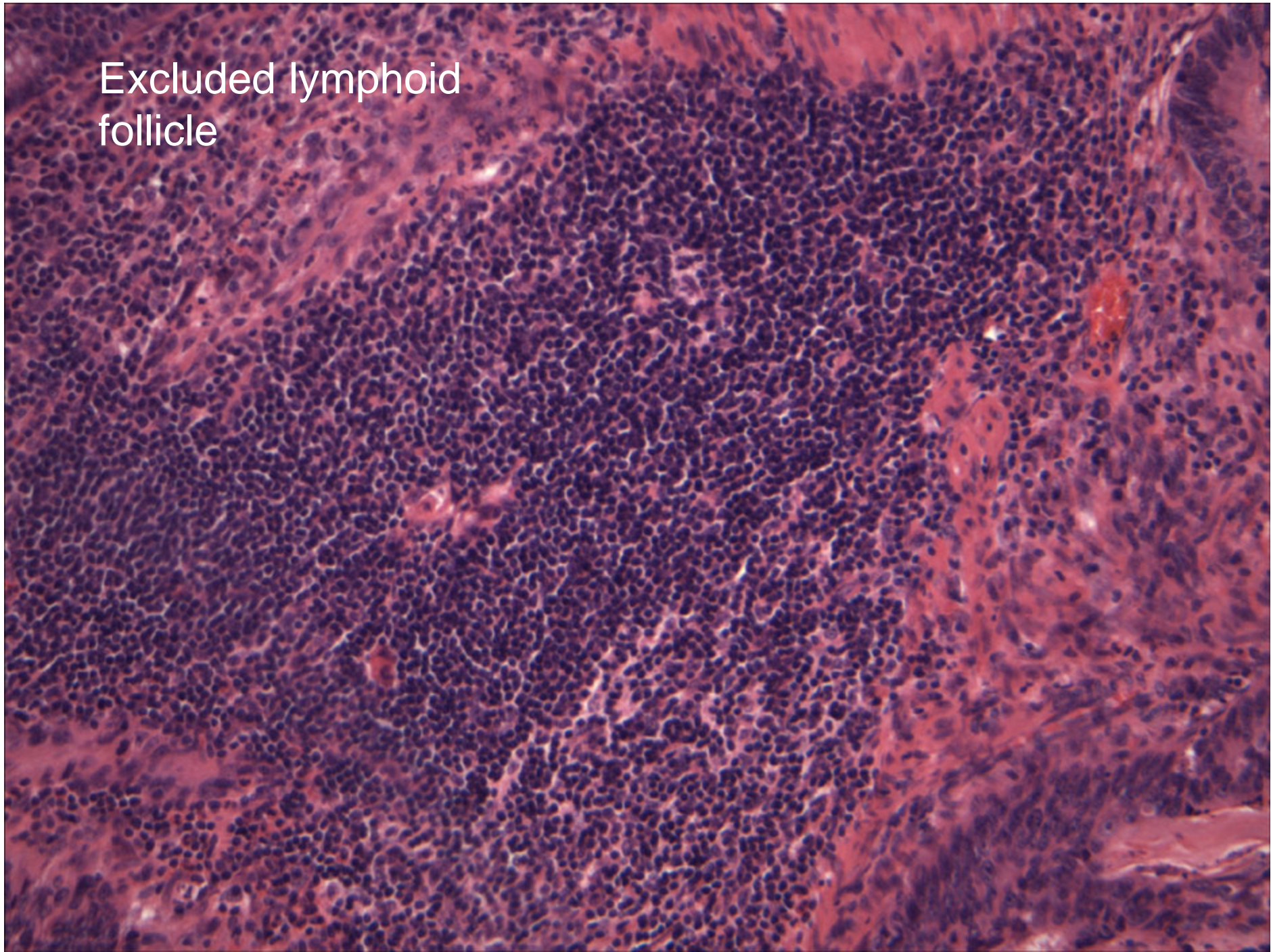


Circled area  
avoids  
lymphoid  
follicle



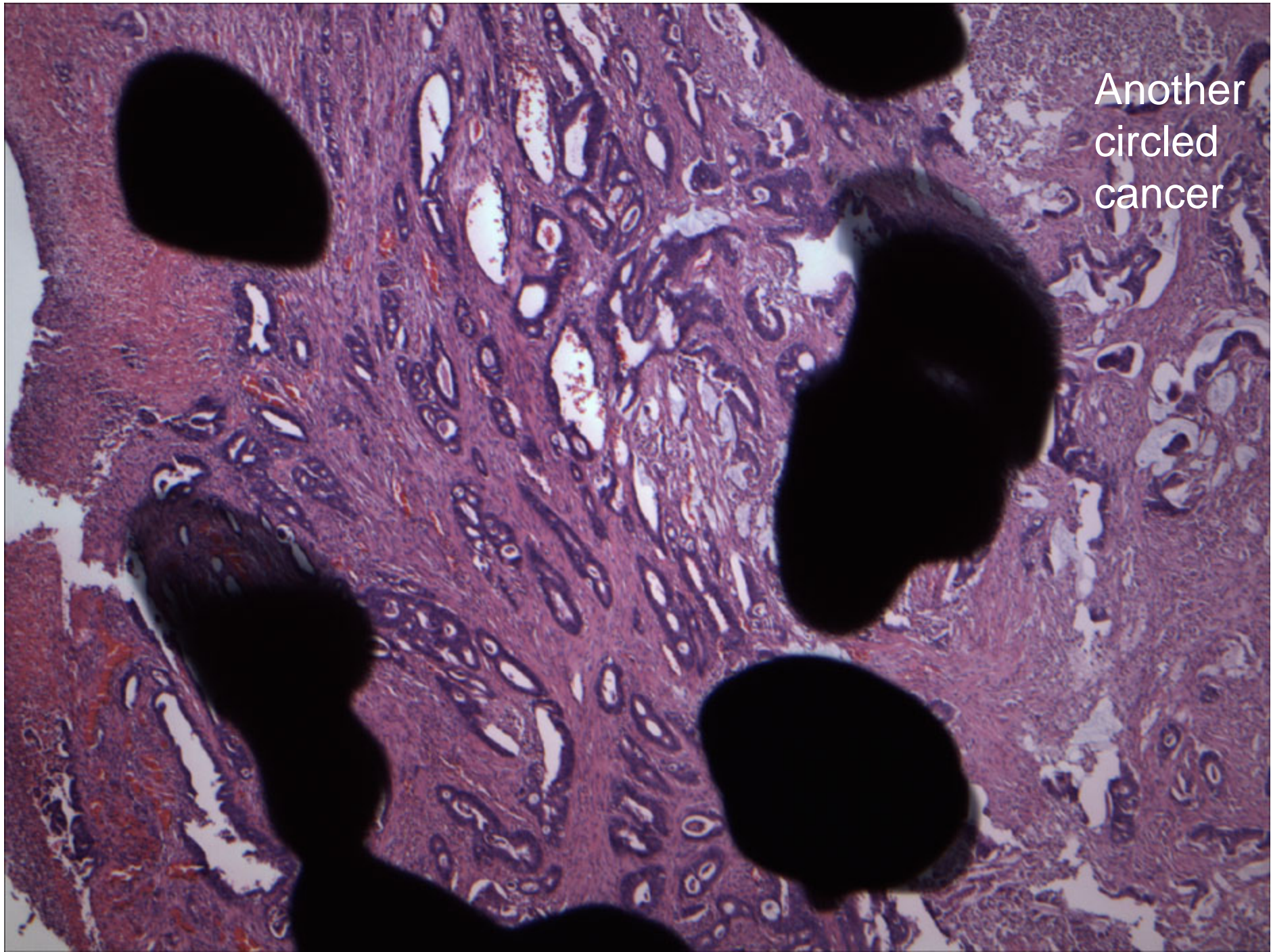


Excluded lymphoid  
follicle

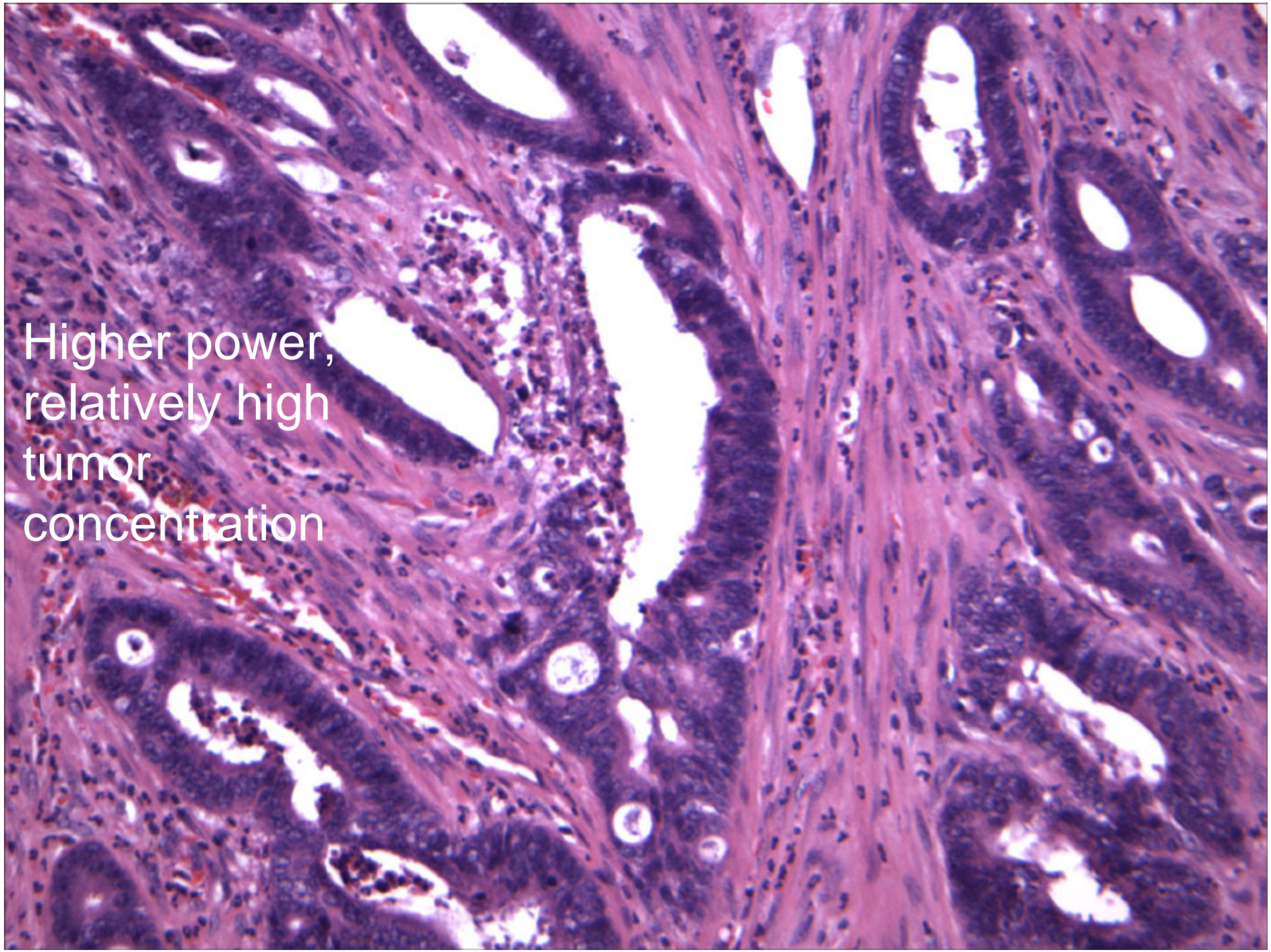




Another  
circled  
cancer







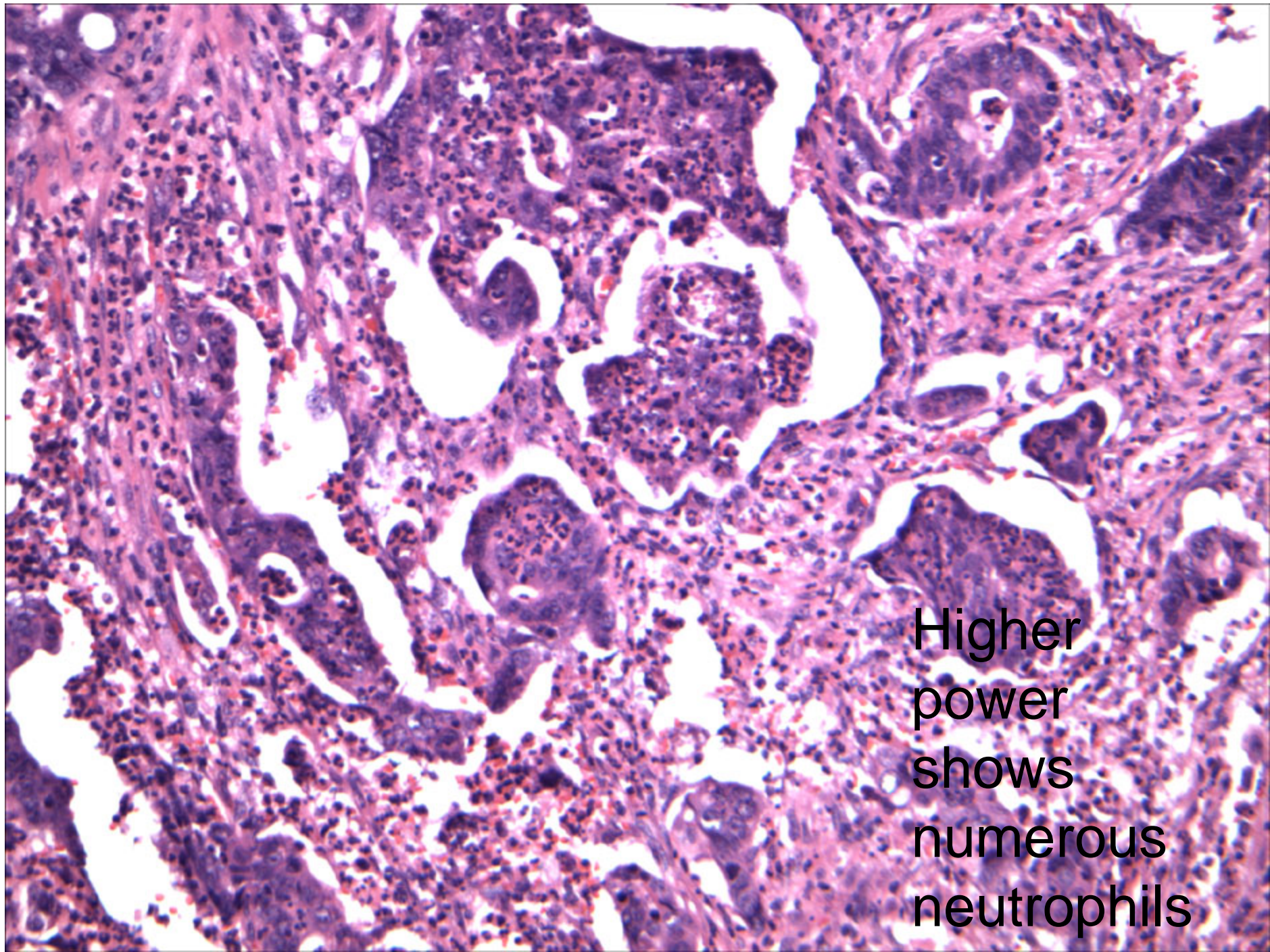
Higher power,  
relatively high  
tumor  
concentration



A histological section of endometrial tissue stained with hematoxylin and eosin (H&E). The image shows a dense population of glands and stroma. A black line is drawn around a specific region in the lower half of the image, which is labeled as 'Another circled area'. The glands within this circled area appear somewhat irregular and crowded. There are also several large, dark, irregular shapes in the upper left quadrant of the image, which appear to be artifacts or shadows.

Another  
circled area





Higher  
power  
shows  
numerous  
neutrophils

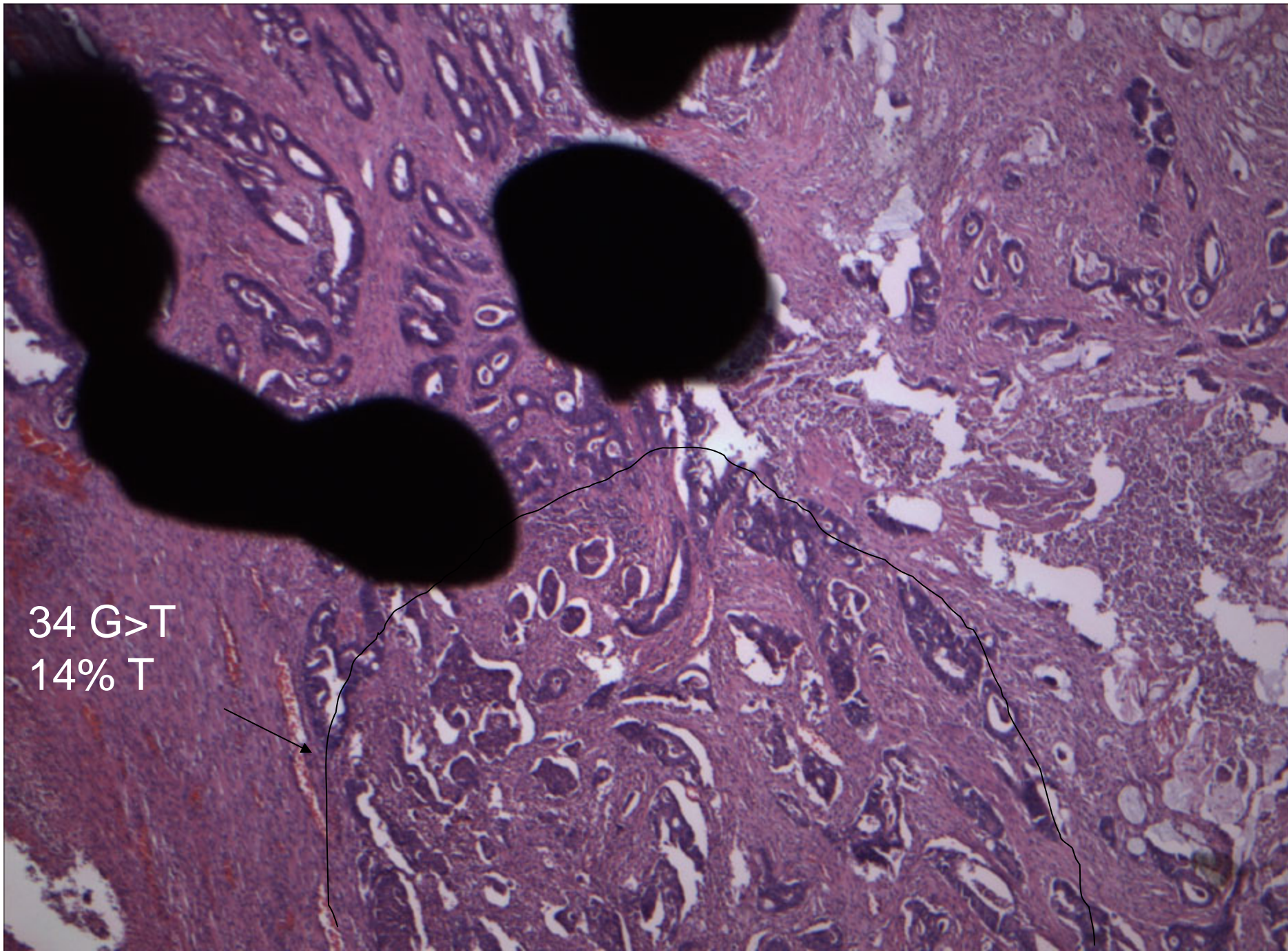




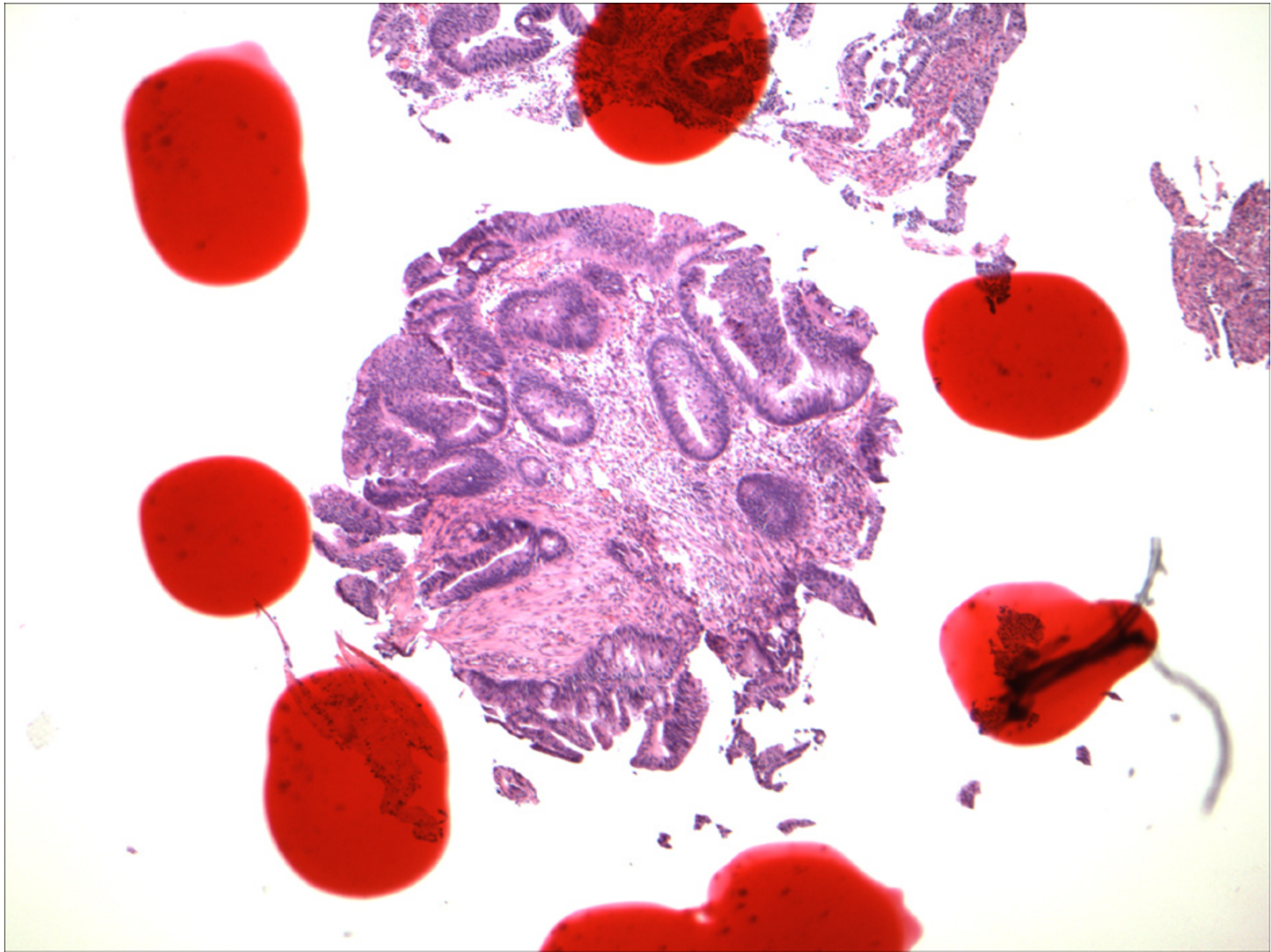
KRAS  
34 G>T  
30%T



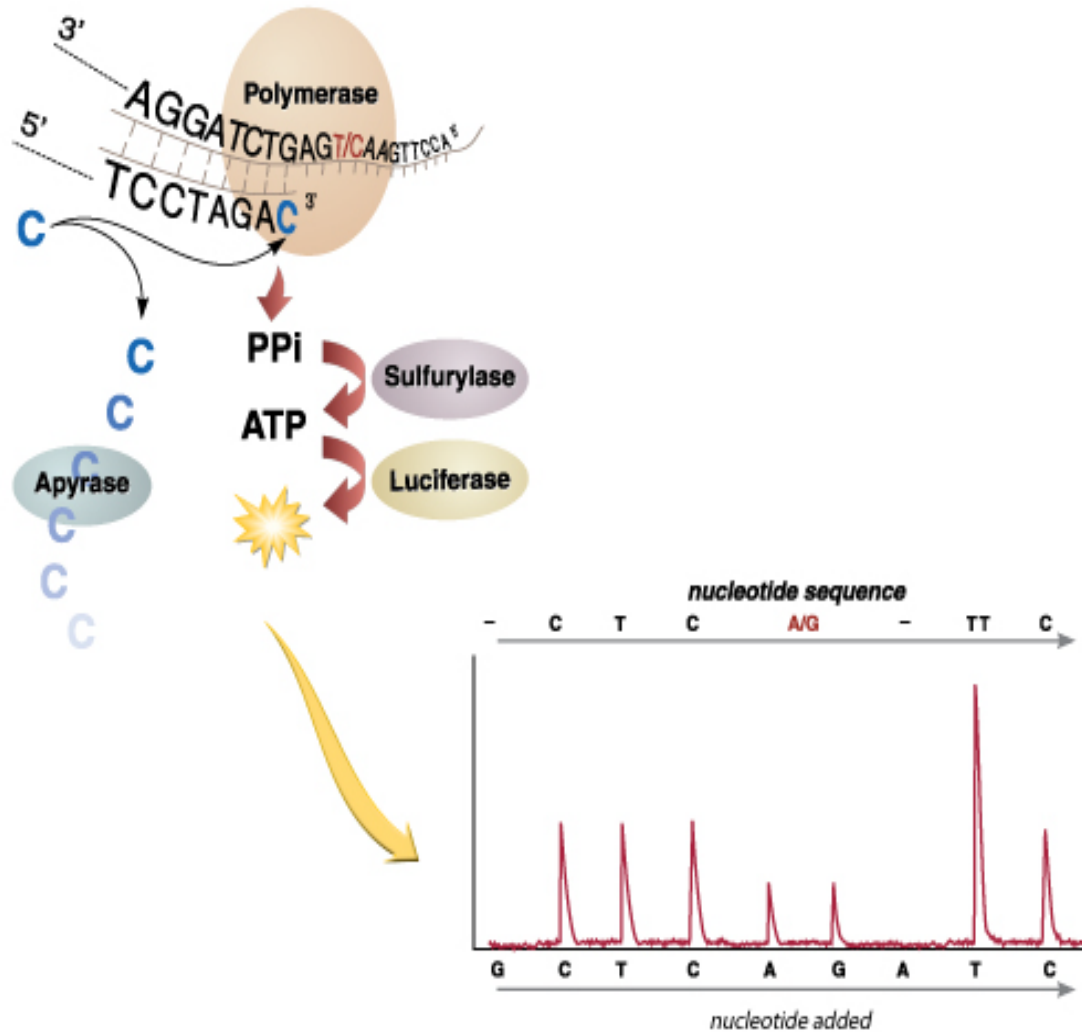
34 G>T  
14% T



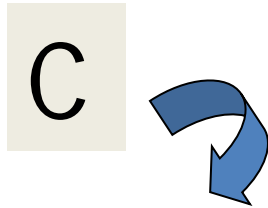




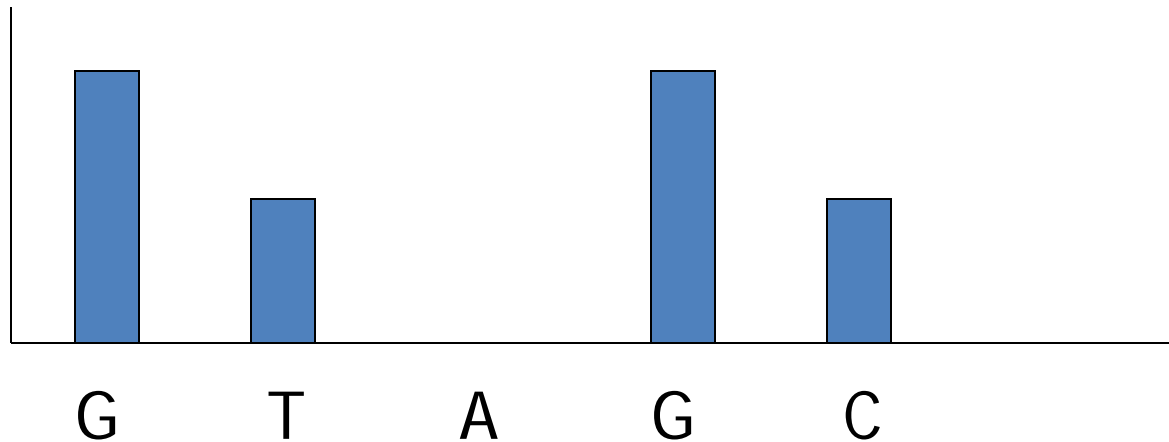
# Pyrosequencing Technology



# PCR and Sequencing Primers

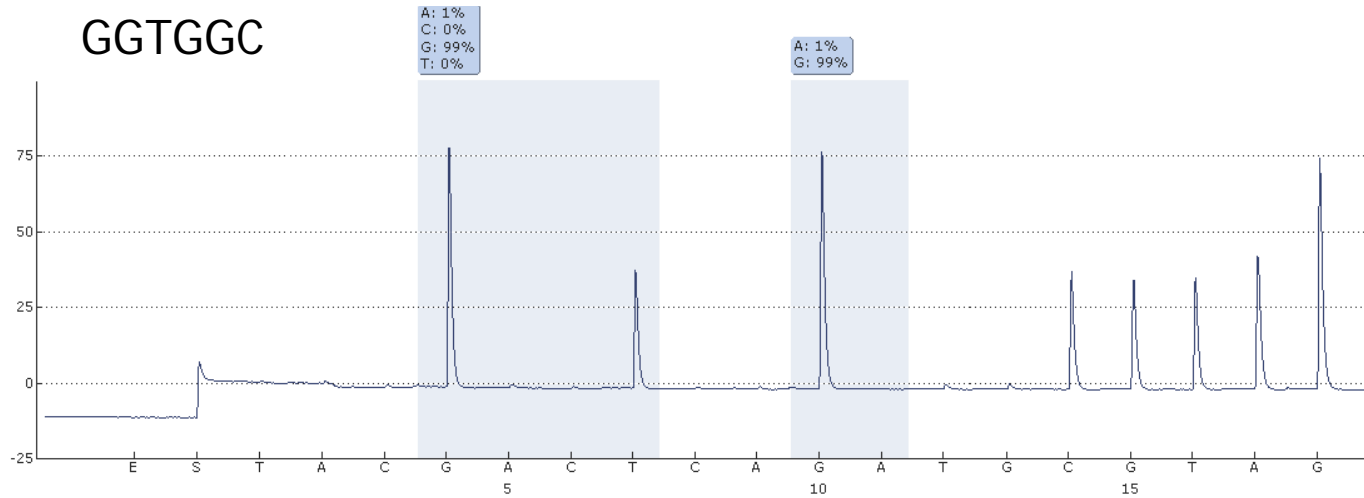


→ GGTGGC  
CCACCGCATCC

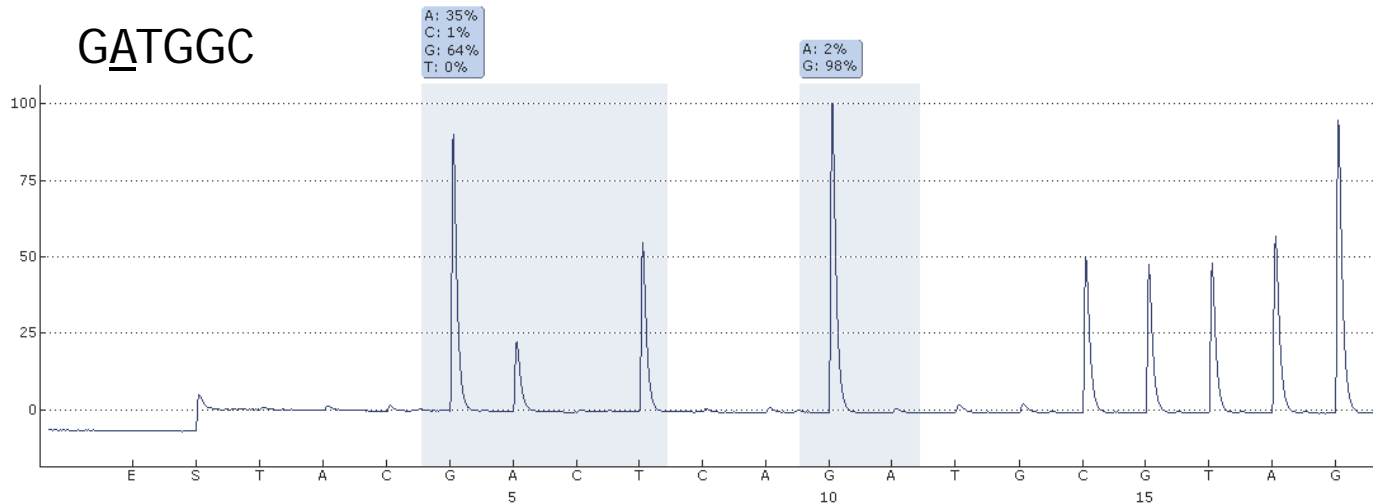


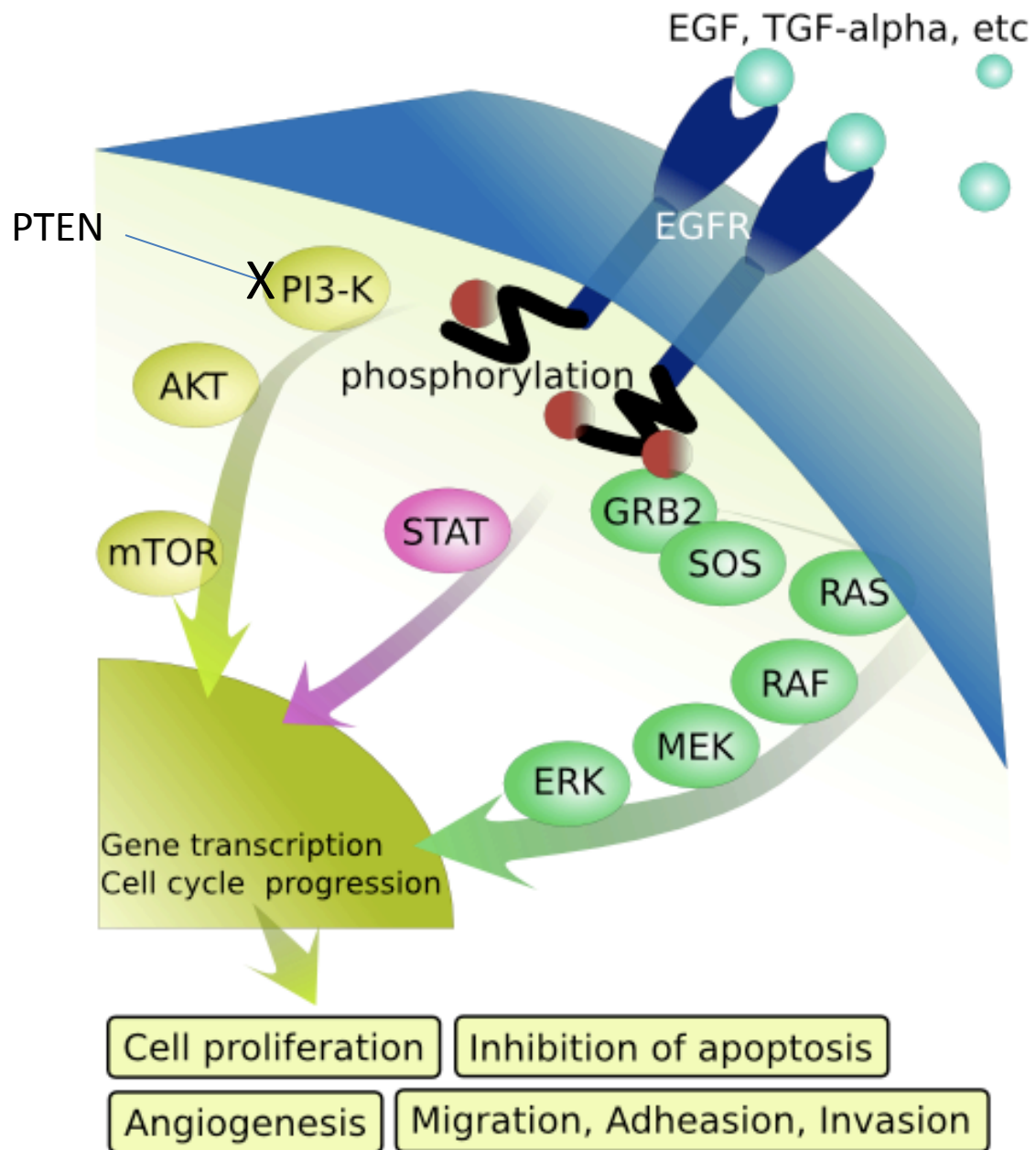
# Pyrosequencing Data Interpretation

Normal



Patient  
c.35G>A,  
p.G12D





Wikimedia



# EGFR pathway inhibition

- EGFR inhibitors used in Stage IV cancers
- Original studies: EGFR inhibition ineffective if mutation in codon 12 or 13 of KRAS
- Subsequently extended to codons 12, 13, 61, 117 or 146 of KRAS and NRAS
- Exon 20 PIK3CA mutations, loss of PTEN
- BRAF may be prognostic marker (bad) rather than predictive of therapy response

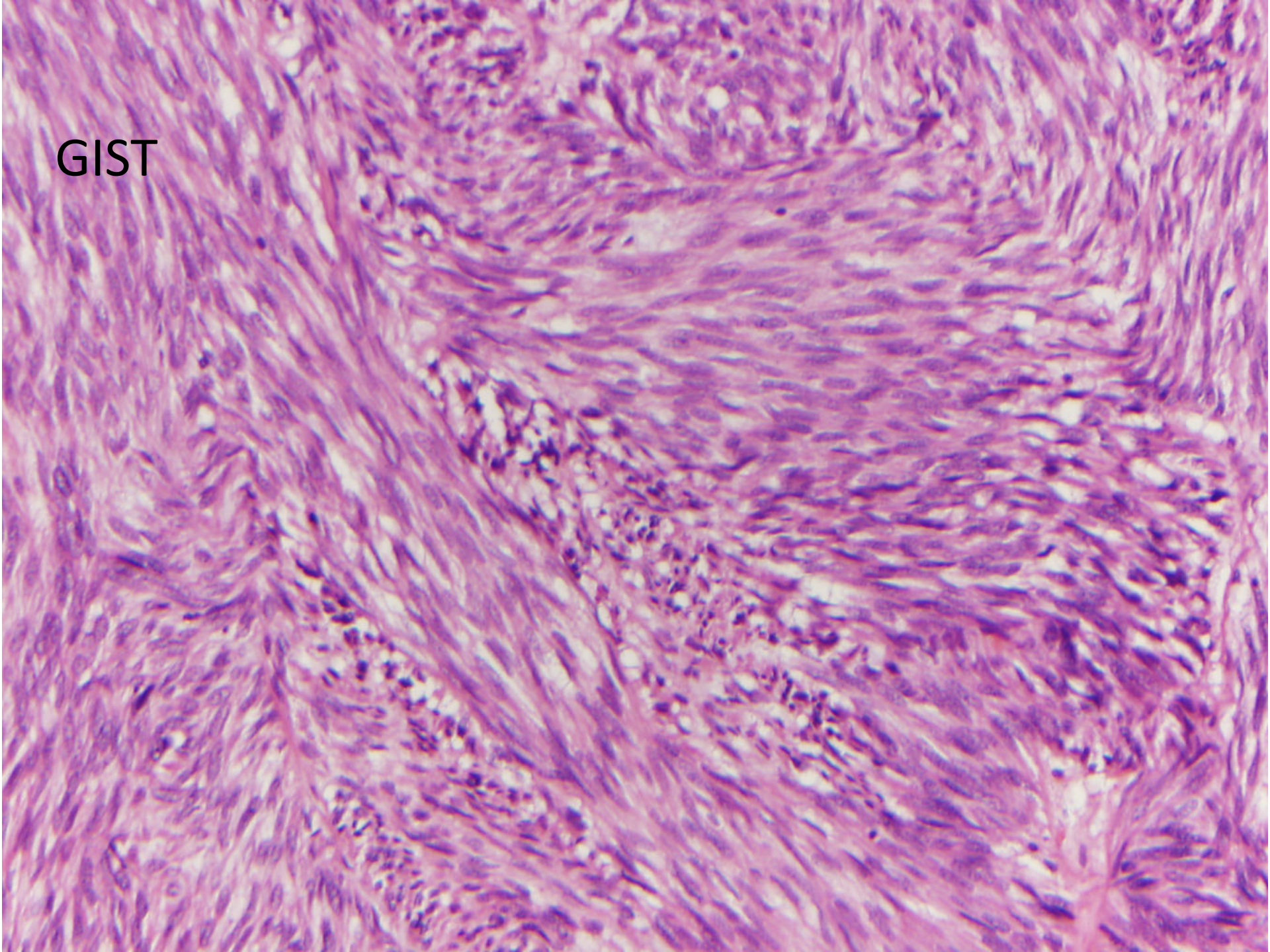
# Case 1

- 70 year old man with metastatic rectal cancer
- Oncologist wishes to treat with cetuximab, orders KRAS testing
- Patient received preoperative chemoradiation; initial slides of resection show pools of mucin with rare malignant glands
- The pre-treatment biopsy is requested
- Pyrosequencing revealed KRAS c.35G>A, p.G12D
- Cetuximab, a very expensive and fairly toxic therapy, was not used

# Case 2

- 61 year old man with a gastric GIST
- We are asked to evaluate tumor for KIT and PDGFRA mutations

GIST

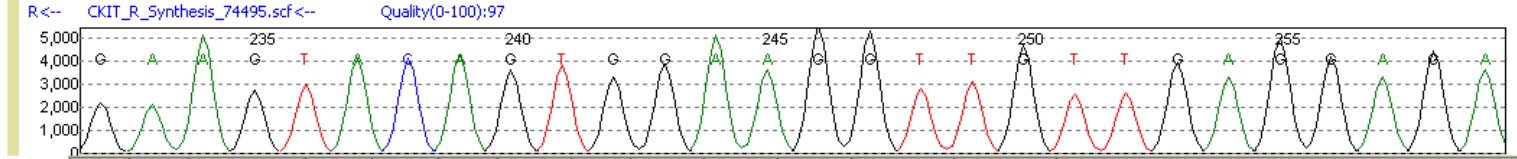
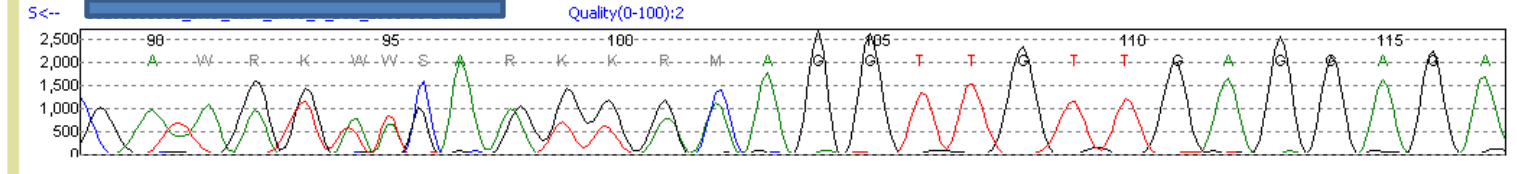
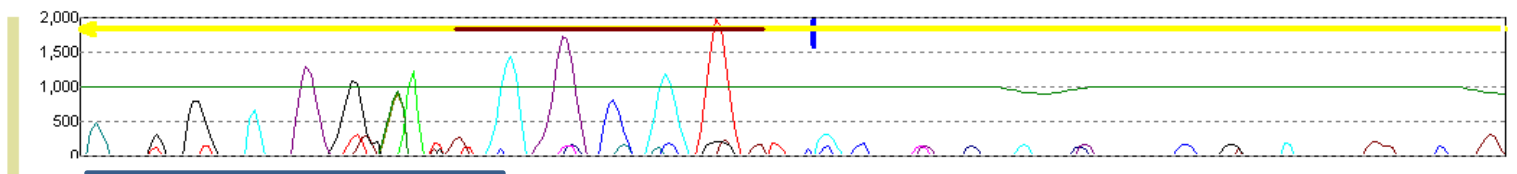
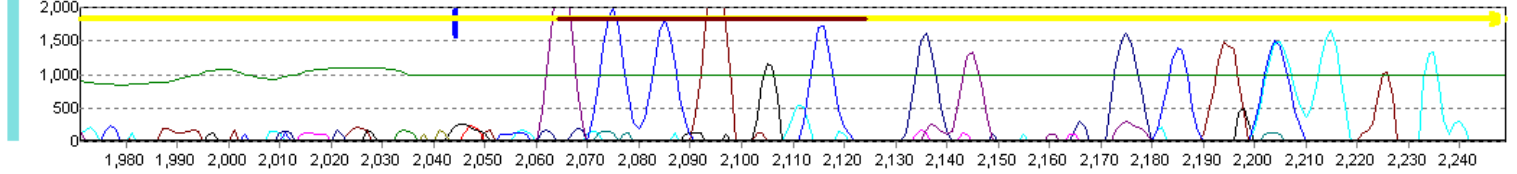
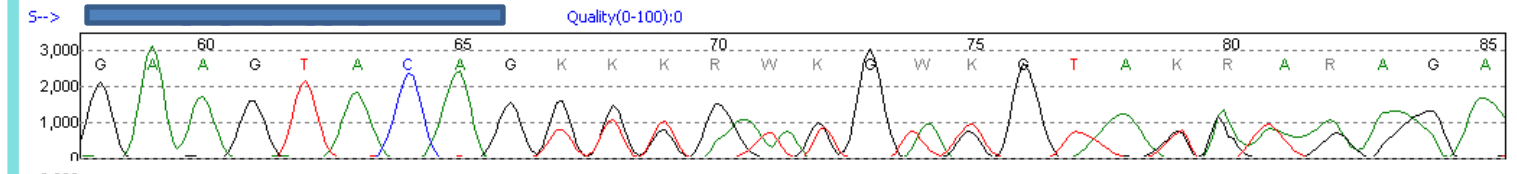
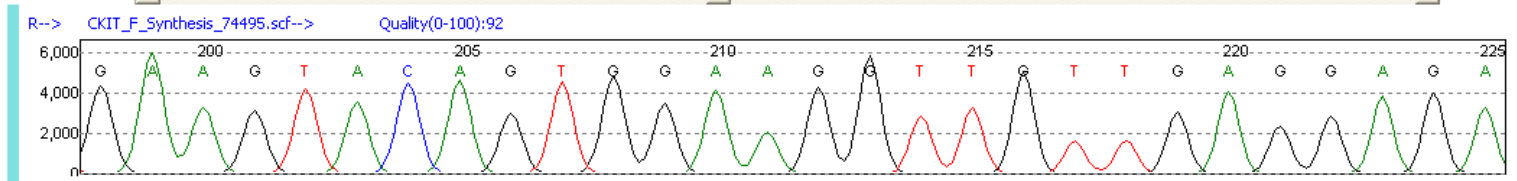




- S:\Anatomic Pathology
  - File
    - GenBank File
    - Reference File
    - Sample File
  - Contig
    - Contig1F (1)
    - Contig1R (1)
    - Contig2F (1)
      - CKIT\_F\_Synt
      - CKIT.gbkl-->
      - 132901063E
    - Contig2R (1)
      - CKIT\_R\_Synt
      - CKIT.gbkl-->
      - 132901063E
    - Contig3F (1)
    - Contig3R (1)
    - Contig4F (1)
    - Contig4R (1)
    - Contig5F (1)
    - Contig5R (1)
    - Contig6F (1)
    - Contig6R (1)
    - Mutation Report

\*KIT.gbkl  
\*-24.ab1

E V555 Q W K V 560 E E  
E V Q W K V V E E



Mut#	Frame	Mutation	mRNA	Intensy	Area	Drop	Overlap	Noise	SnRatio	Score	Total Score	Comments
1	2045	74441_74446he c.1754_1										
2	2065	74443_74448he c.1756_1										

KIT, Exon 11, c.1669\_1674del, p.W557\_K558del

# What's a GIST?

- Smooth muscle? Leiomyoma, Leiomyosarcoma
- Neural? Schwannoma?
- Unknown: Stromal tumor
- CD 117 (KIT) positive in 95%
- KIT: type III receptor tyrosine kinase expressed in
  - Interstitial cells of Cajal
  - Melanocytes
  - Mast cells
  - Germ cells



# Where are GIST's?

- Stomach: 50%
- Small intestine: 25%
- Esophagus, colon, rectum: 10%
- Extra-intestinal (mesentery, omentum, retroperitoneum): 10%

# GIST: benign, malignant or **stratify risk?**

- Use mitotic rate and size to estimate risk of progressive disease
  - Very low risk, low risk, intermediate risk, high risk (Fletcher, Hum Pathol 2002;33:459-65)
  - Doesn't apply to succinate dehydrogenase deficient GIST's
- Factor in location, as gastric generally does better than small intestine or rectum (Miettinen, Semin Diagn Pathol 2006; 23:70-83)

# What genes are mutated in GIST's?

- KIT: 80% of GISTS
- PDGFRA: 8%
- KIT and PDGFRA mutations are mutually exclusive
- “Wild type” (No Kit or PDGFA mutation): 10-15%
  - Half are succinate dehydrogenase deficient
    - Some of these have germline mutations
  - Rare: BRAF mutation

# KIT and PDGFRA

- Homologous type III receptor tyrosine kinases
- Extracellular domain (5 IG like domains), transmembrane sequence, juxtamembrane domain, split tyrosine kinase

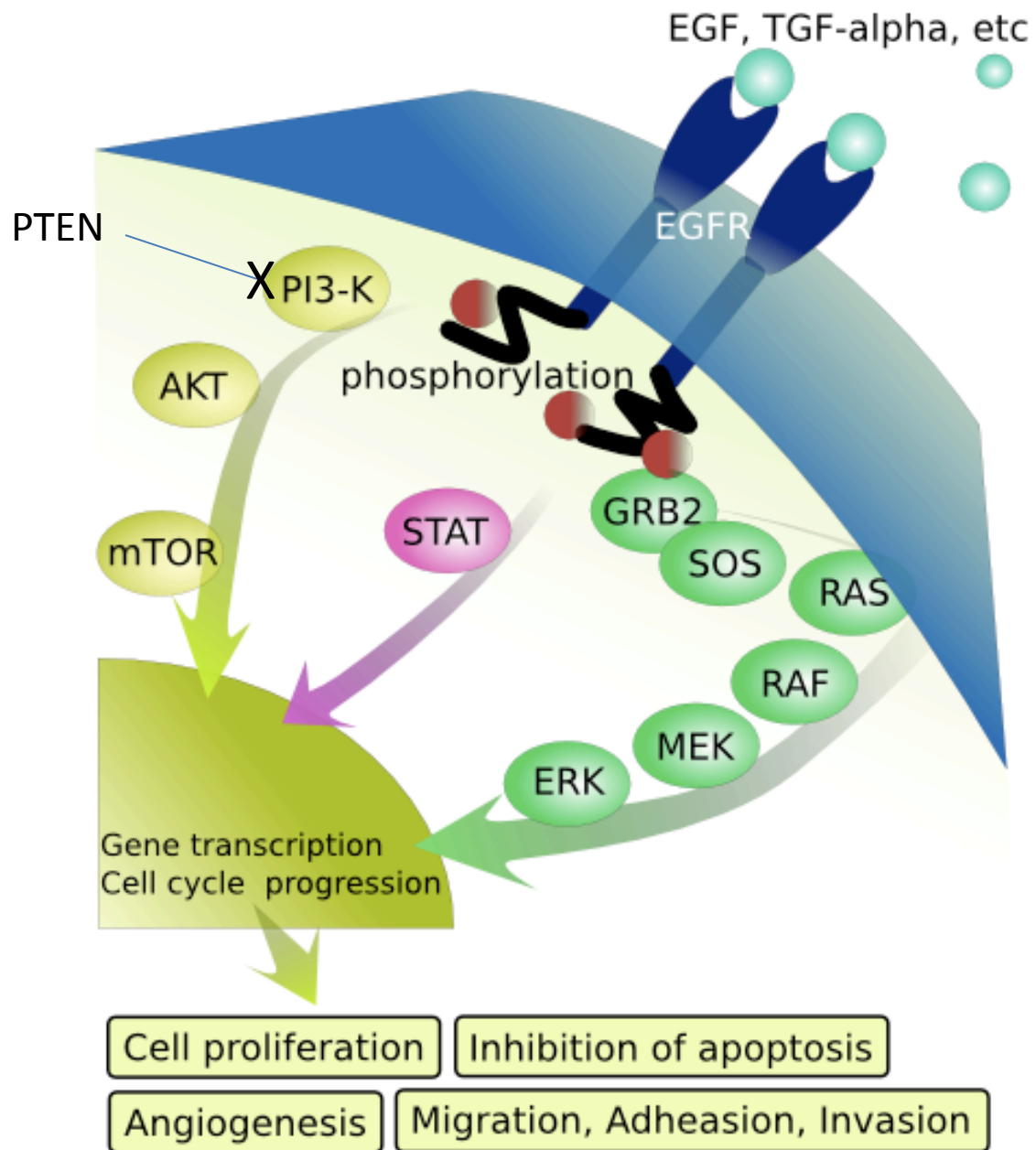


# What are kinases?

- Transfers phosphate, usually from ATP, to a substrate, aka phosphorylation
- Protein kinases phosphorylate certain amino acids, like tyrosines, serines, or threonines
- Activates or transmits a signal in a pathway
- Uncontrolled activation may be oncogenic
- Kinase inhibitors block phosphorylation and inhibit tumor progression

# Important kinases in tumors

- EGFR, ROS, RET, ALK, KIT, PDGFRA, HER2: receptor tyrosine kinases
  - Extracellular ligand binding
  - Transmembrane region
  - Intracellular kinase domain that phosphorylates tyrosines (both its own and other proteins)
- BRAF, MTOR, AKT: serine/threonine kinases
- PIK3CA: lipid kinase upstream of AKT
- PTEN: **not a kinase**, rather a phosphatase that dephosphorylates target of PIK3CA



Wikimedia

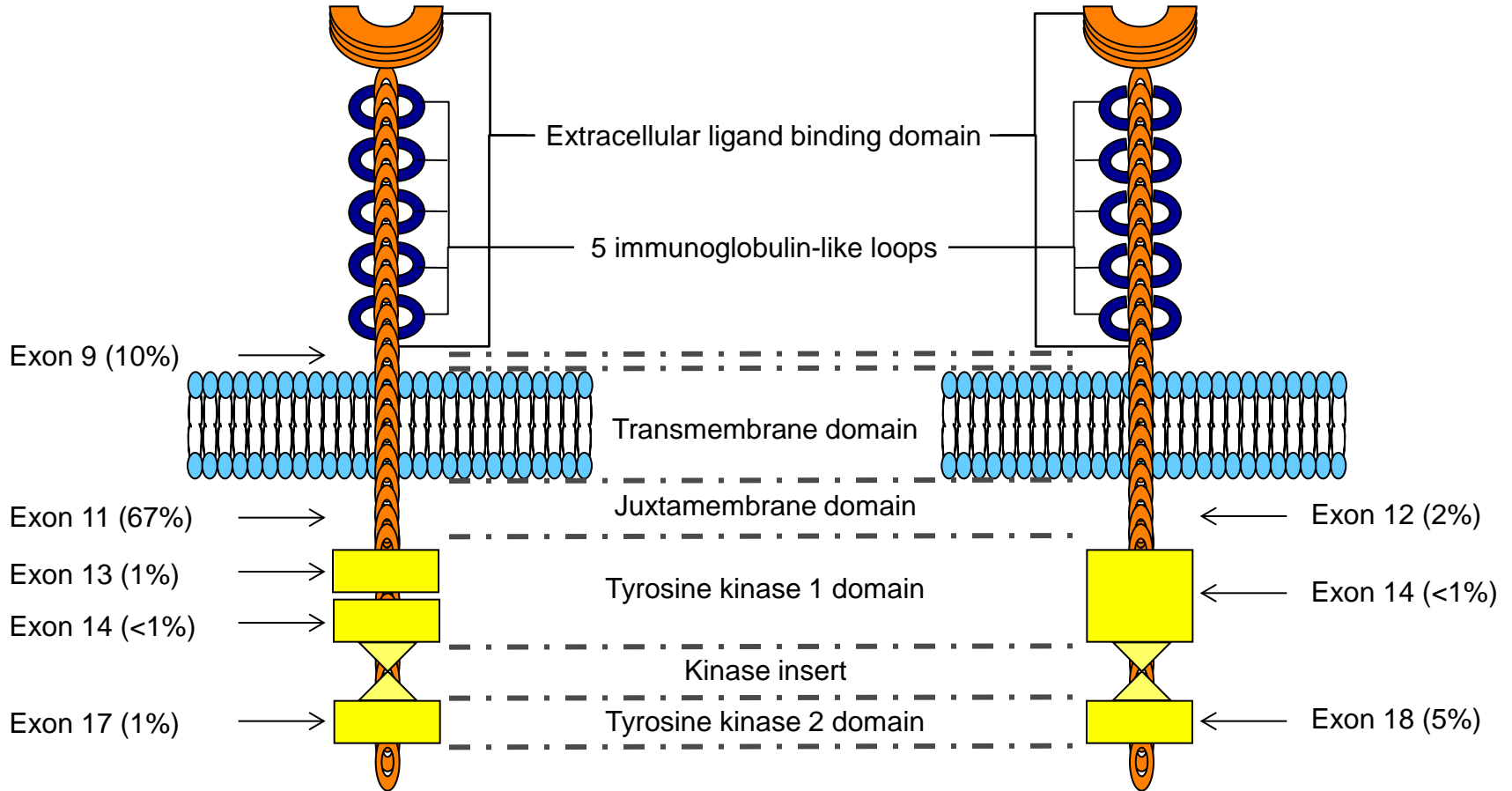
# How are kinases activated in tumors?

- Tyrosine kinase domain mutations (EGFR)
- Ligand independent receptor dimerization (KIT)
- Translocations fusing the tyrosine kinase domain to another gene (EML4-ALK)
- Amplification (HER2)



# KIT

# PDGFRA



# KIT and PDGFR Mutations in GIST

- KIT: 80% of GISTS
  - Exon 9 (extracellular): 10%
  - Exon 11 (juxtamembrane): 67%
  - Exon 13 (kinase I): 1%
  - Exon 17 (kinase II): 1%
- PDGFR (homologous RTK): 8%
  - Exon 12 (juxtamembrane): 2%
  - Exon 14 (kinase I): <1%
  - Exon 18 (kinase II): 5%
  - No exon 10 (extracellular) mutations

# How do KIT mutations cause tumors?

- Mutations in extracellular or juxtamembrane domains (exons 9 and 11) lead to ligand independent receptor dimerization and activation
- Primary TK2 (exon 17) mutations stabilize activation loop in active configuration
- Unclear how primary TK1 (exon 13) mutations are oncogenic; maybe interfere with juxtamembrane domain inhibition of activation loop
- Secondary (after drug treatment) TK mutations important for drug resistance

# Mutations and risk stratification

- Currently not included
- Mutation-risk relationships do exist, but
  - Micro-gists (1-10mm in size) in up to 35% extensively sampled stomachs
  - Vast majority do not progress
  - Type and frequency of Kit mutations the same as for clinically relevant lesions
  - PDGFRA mutations also seen
- Therefore, mutational status cannot be considered independent of other risk factors



# KIT exon 9 and 11 mutations

- In frame insertions, deletions, duplications, substitutions, or combinations
- More than 90 exon 11 mutations reported
  - Most are deletions (cluster at 5 prime end, duplications at 3 prime)
  - p.W557\_K558 deletion most common (gastric)
  - p.Y568del, p.Y570 deletion small intestine
  - Deletions in general, and p.W557del and/or K558 deletion in particular, associated with worse prognosis
- Exon 9 small intestine and colon, more aggressive
  - Requires higher dose imatinib
  - p.A502\_Y503dup most common mutation
- 15% Kit mutations are homozygous, more aggressive

# Tyrosine Kinase KIT mutations

- Substitutions more common than deletions, insertions
- Exon 13 (TK1)
  - p.K642E most common mutation
- Exon 17 (TK2)
  - Codon 822 substitution most common

# PDGFRA mutated GIST's

- Epithelioid morphology
- Gastric and extra-GI location
- Kit negative (or weakly positive) by IHC
- May be less aggressive
- D842V in TK2 is most common mutation



# Treatment

- Surgery first line therapy
- Imatinib competes with ATP for binding site, works against non-TK mutations
- Inhibits KIT and PDGFRA and is used for metastatic disease, when surgery is not an option, or after surgery with high risk of recurrence
- Kit exon 11 mutated tumors more likely to respond to imatinib than exon 9 mutated or wild type
- Kit exon 9 mutated tumors respond better to higher dose of imatinib

# Imatinib resistance

- Primary Resistance: Kit WT, KIT exon 9 mutants (may be function of dose), PDGFRA p.D842V
- Secondary resistance: secondary mutations in KIT exons 13, 14 (TK1) which interfere with drug binding and 17,18 (TK2) which stabilize TK2 in active conformation
  - Usually single nucleotide substitutions
  - Occur on same allele as original mutation
- Secondary mutations more likely to occur in exon 11 mutated tumors than exon 9 (possibly dose related)
- Secondary mutations not seen in wild type tumors

# Therapy for resistant tumors

- Sunitinib (second generation TKI) used for those who fail imatinib, active against ATP binding pocket mutations
- Many alternative TKI's target VEGF
- PDGFRA p.D842V is resistant to both TKI's
  - May be sensitive to Dasatinib



# GIST syndromes

- Familial GIST: Multiple tumors, diffuse hyperplasia of interstitial cells of Cajal, mastocytosis
  - KIT and PDGFRA germline mutations
- NF1: 7% multiple small intestinal GIST's, do not metastasize, no KIT or PDGFRA mutations
- Carney's triad (not inherited)
  - Pulmonary chondroma, extra-adrenal paraganglioma and epithelioid gastric GIST, mostly young women
  - No KIT or PDGFRA mutations
  - Succinate dehydrogenase deficient (but no mutations)
- Carney-Stratakis syndrome
  - Multifocal gastric GISTS and paraganglioma
  - Germline mutations in succinate dehydrogenase subunits

# Succinate dehydrogenase

- Complex of 4 proteins: SDHA,B,C,D
- Inner mitochondrial membrane but coded by nuclear DNA
- Germline mutations first noted in hereditary paraganglioma
- Possible mechanism: Succinate accumulation drives HIF1a (hypoxia-inducible factor 1 alpha) overexpression then insulin-like growth factor 2 and VEGF activation

# SDH-deficient GIST's

- Half of wild type GIST's, 7.5% of gastric
- Most pediatric GIST's
- Despite lymph node (unusual for GIST's) and distant metastases, indolent behavior
- Accepted GIST risk factors don't apply
- Distinctive morphology: epithelioid, multinodular/plexiform
- Nearly all KIT and DOG-1 positive
- Do not respond to imatinib

# SDH-deficient GIST's

- Regardless of subunit mutation, tumor will show loss of cytoplasmic staining for SDHB (good screening tool for SDH-deficient)
- Minority due to germline mutations in SDH B, C or D (Carney-Stratakis syndrome)
- New: about 30% (?) due to apparent germline mutations in SDHA (loss of IHC staining for A and B) Miettinen, AJSP, 2013
- SDHA has 3 pseudogenes



- About half of inflammatory fibroid polyps have PDGFRA mutations
  - Negative for Kit and Dog1
  - Benign
  - Lasota, Mod Pathol, 2009

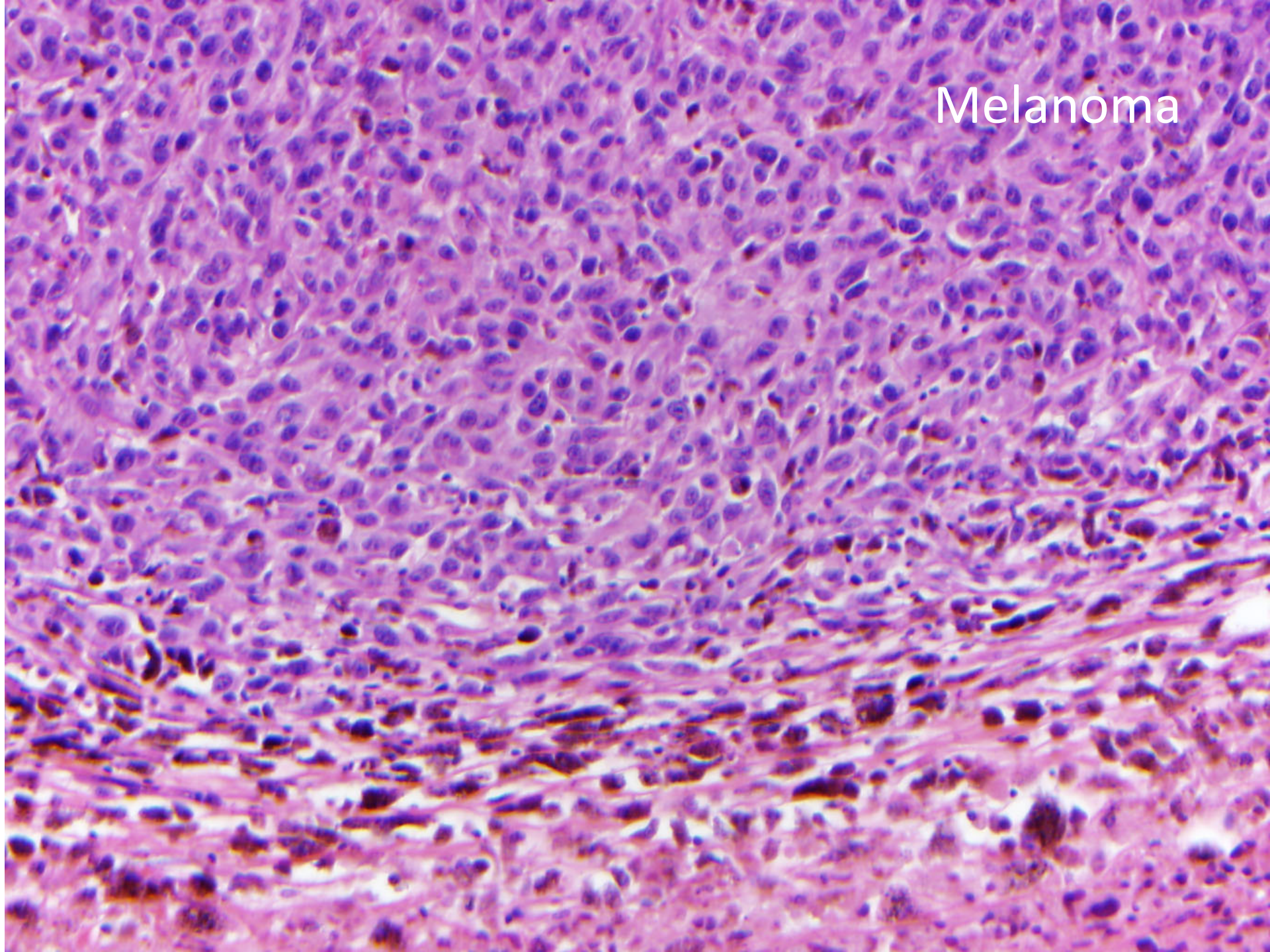
# Case 2

- 61 year old man with a gastric GIST
- We are asked to evaluate tumor for KIT and PDGFRA mutations
- A KIT exon 11, c.1699\_1674del, p.W557\_K558del was detected
- The patient was treated with imatinib with a good response

# Case 3

- 43 year old woman with metastatic melanoma
- BRAF, NRAS and c-KIT mutation testing is ordered.

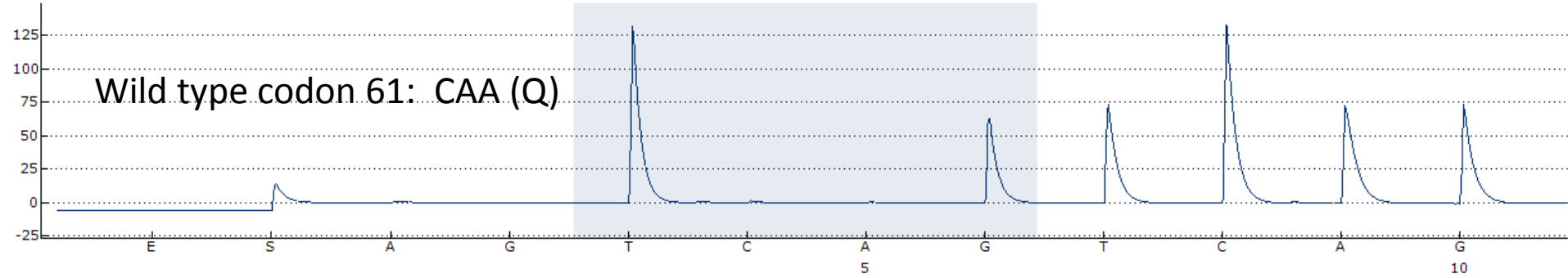
Melanoma





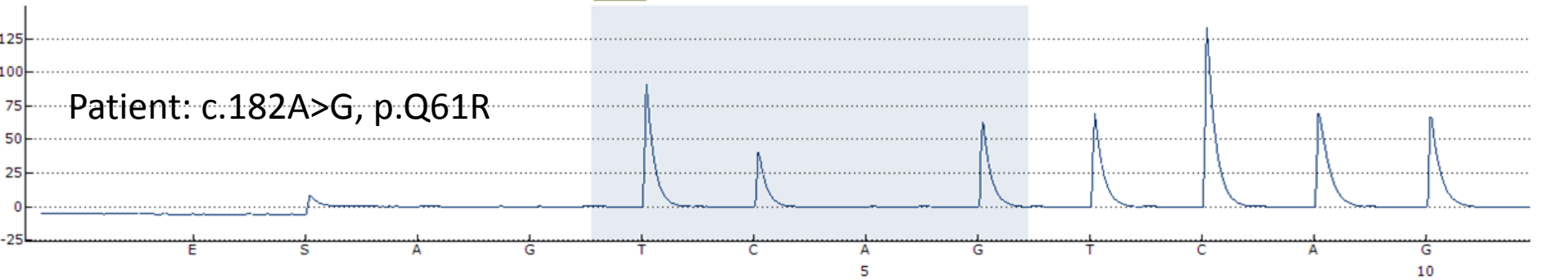
A: 0%  
C: 0%  
G: 0%  
T: 100%

Wild type codon 61: CAA (Q)



A: 0%  
C: 61%  
G: 0%  
T: 39%

Patient: c.182A>G, p.Q61R



# What is most commonly mutated gene in melanoma? BRAF

- BRAF mutations in half of cutaneous melanomas
- Most common on trunk and extremities, areas of intermittent sun exposure
- Less common in chronic sun damaged skin, like the face
- Less common in acral lentiginous (10%) or mucosal (15%) sites
- 80% of benign nevi (not useful in malignancy determination)
- V600E in most, also V600K,R

# BRAF-directed therapy

- Vemurafenib efficacious against V600E, uncertain if it works against V600K,R
- Resistance commonly develops
  - Upregulation of PDGFRB, IGF-1R, CRAF, COT/MAP3K8, MEK, NRAS mutation, PTEN loss
  - Either re-activate MAPK or stimulate AKT pathway
- Side effect: cutaneous squamous cell ca (KA)
  - Paradoxical MAPK stimulation through CRAF
  - Ras mutations (often HRAS, codon 61) in tumors

# BRAF-directed therapy

- Contraindicated for BRAF wild type tumors
  - Doesn't work
  - Paradoxical activation of MAPK signaling may accelerate disease progression
  - Squamous cell cancers and other toxicities



# NRAS mutations

- Second most common mutation in melanoma (15-25%)
- Codon 61 mutations are most common
  - Q61K, R, H
- MEK inhibitors in clinical trials (Ascierto, Lancet Oncol, 2013; Grimaldi, Curr Opin Oncol, 2014)

# Kit mutations

- More common in acral lentiginous (11-38%), mucosal (6-19%), and chronic sun damaged skin (17%)
- Most are sensitive to imatinib
- Mostly exon 11, 34% L576P
- Less commonly 13, 17 or 18
- Some controversy, but evidence that CD117 IHC doesn't predict mutation status

# Case 3

- 43 year old woman with metastatic melanoma
- BRAF, NRAS and c-KIT mutations testing is ordered.
- An NRAS c.182A>G, p.Q61R mutation is detected. BRAF and c-KIT are wild type (mutations are mutually exclusive).
- Patient is enrolled in a MEK inhibitor trial.

# Case 4

- 73 year old woman with a right upper lobe lung mass. Bronchoscopic biopsy is performed. Tumor cells are positive for CK7, TTF1 and Napsin A, consistent with a primary lung adenocarcinoma.
- EGFR mutation detection was requested



# EGFR mutations in NSCLC

- Mutated in about 10% of US NSCLC
  - Higher in women, East Asians, never or light smokers
- Mostly in exons 18-21 of tyrosine kinase domain
  - 45% in frame exon 19 deletions
  - 40% L858R in exon 21
  - 2-5% codon 719 in exon 18
  - 1-2% codon 768 in exon 20
  - 2% codon 790 in exon 20 (T790M)
  - 5-10% in frame exon 20 insertions
  - 2-5% codon 861 in exon 21

# EGFR and TKI therapy

- Small molecule TKI's only work against tumors with EGFR tyrosine kinase mutations
- Mutated allele often amplified, but mutation, not amplification, determines TKI response
- T790M mutation is resistant (most common secondary resistance mutation after TKI therapy)
- Rarely T790M is initial mutation, often germline in origin, often coupled with another EGFR mutation
- Exon 20 insertions resistant to TKI's, except for A763\_Y764insFQEA (Yasuda, 2013)

# Other mutations

- KRAS: 25% of NSCLC, resistant to TKI's
- EML4-ALK rearrangement in 2-7% NSCLC
  - Responds to crizotinib
  - Detected by ALK antibody clone D5F3, FISH
- ROS1 rearrangements in 1-2%
  - Responds to crizotinib
  - Antibody, FISH
- RET rearrangements in 1-2% (Wang, JCO, 2012)
- EGFR and the above mutations are typically mutually exclusive

# Next generation sequencing (NGS)

- Cost effective way to test multiple genes
  - And gene list for lung is growing: HER2, PIK3CA, BRAF, NRAS, etc.
- Targeted NGS approach requires very little input DNA (10 ng) and provides fast turnaround time
- Covers the genes we've been talking about today, and many others (48 genes, nearly 3,000 mutations)
- Can be adapted to detect translocations



# Molecular Testing Guidelines

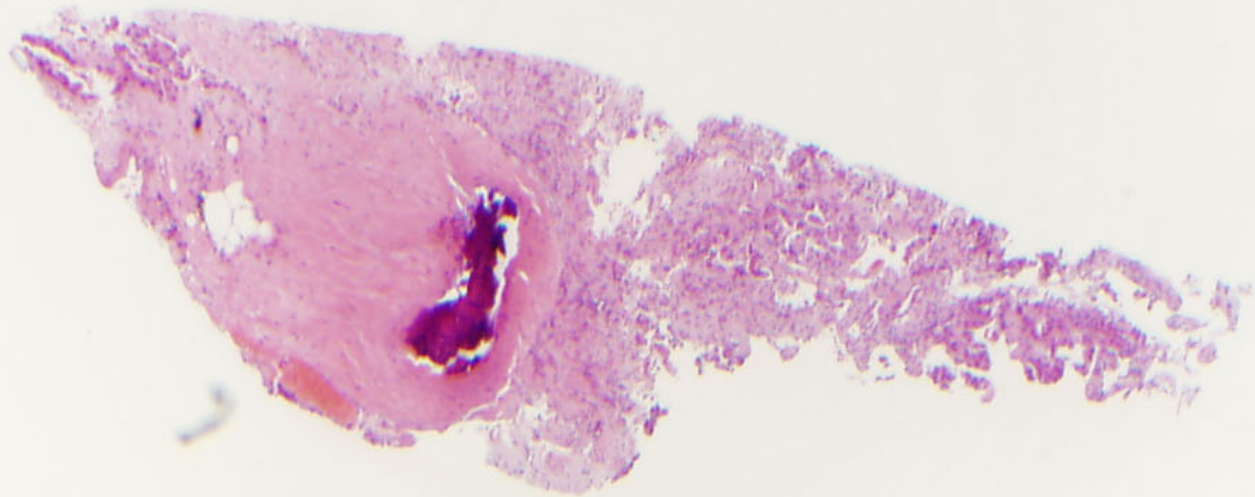
## Lindeman et al, 2013

- 2.3: Tissue should be prioritized for EGFR and ALK testing
  - Don't use up scant tissue with IHC stains
  - Can cut unstained at time of initial diagnosis

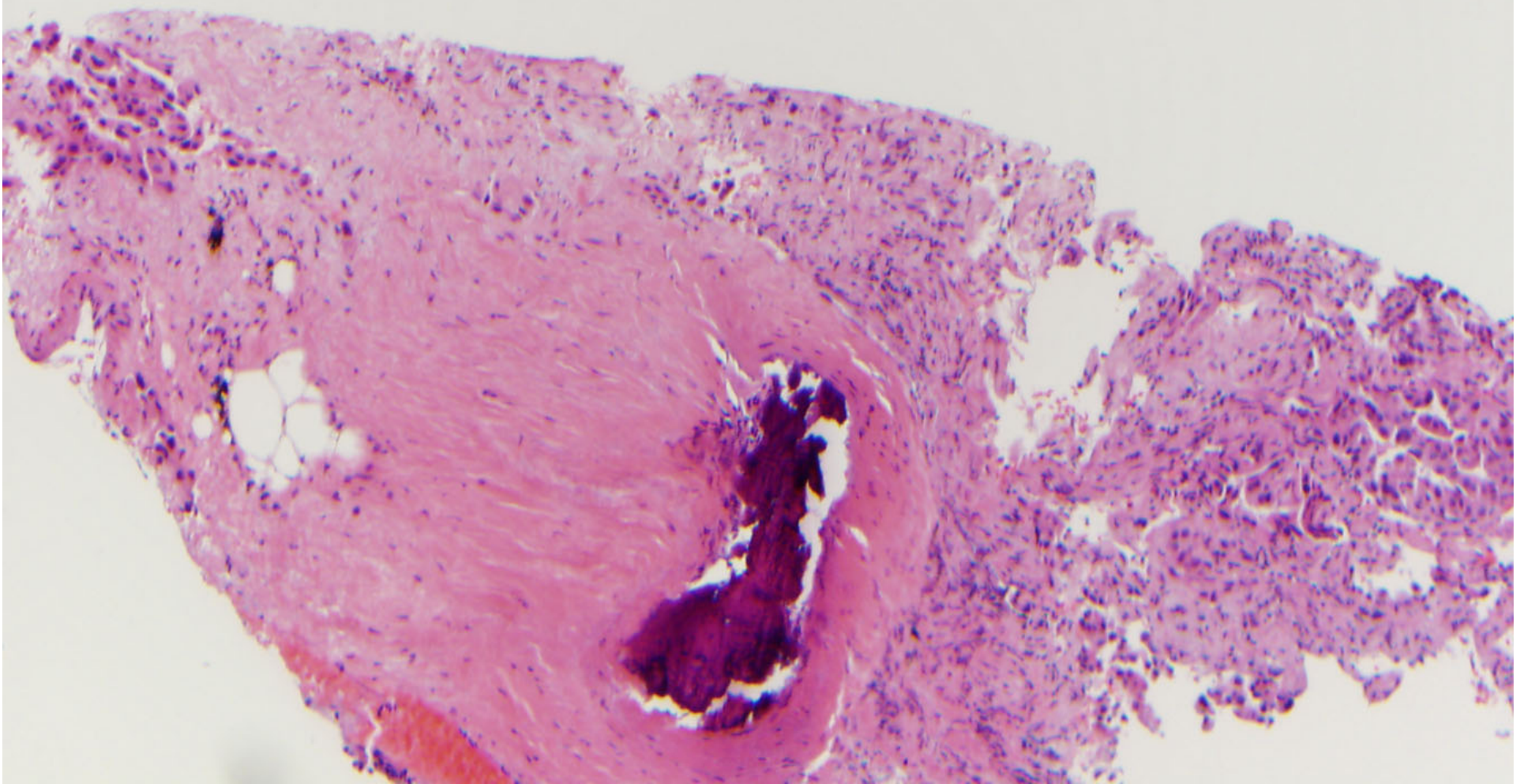
ARUP

14



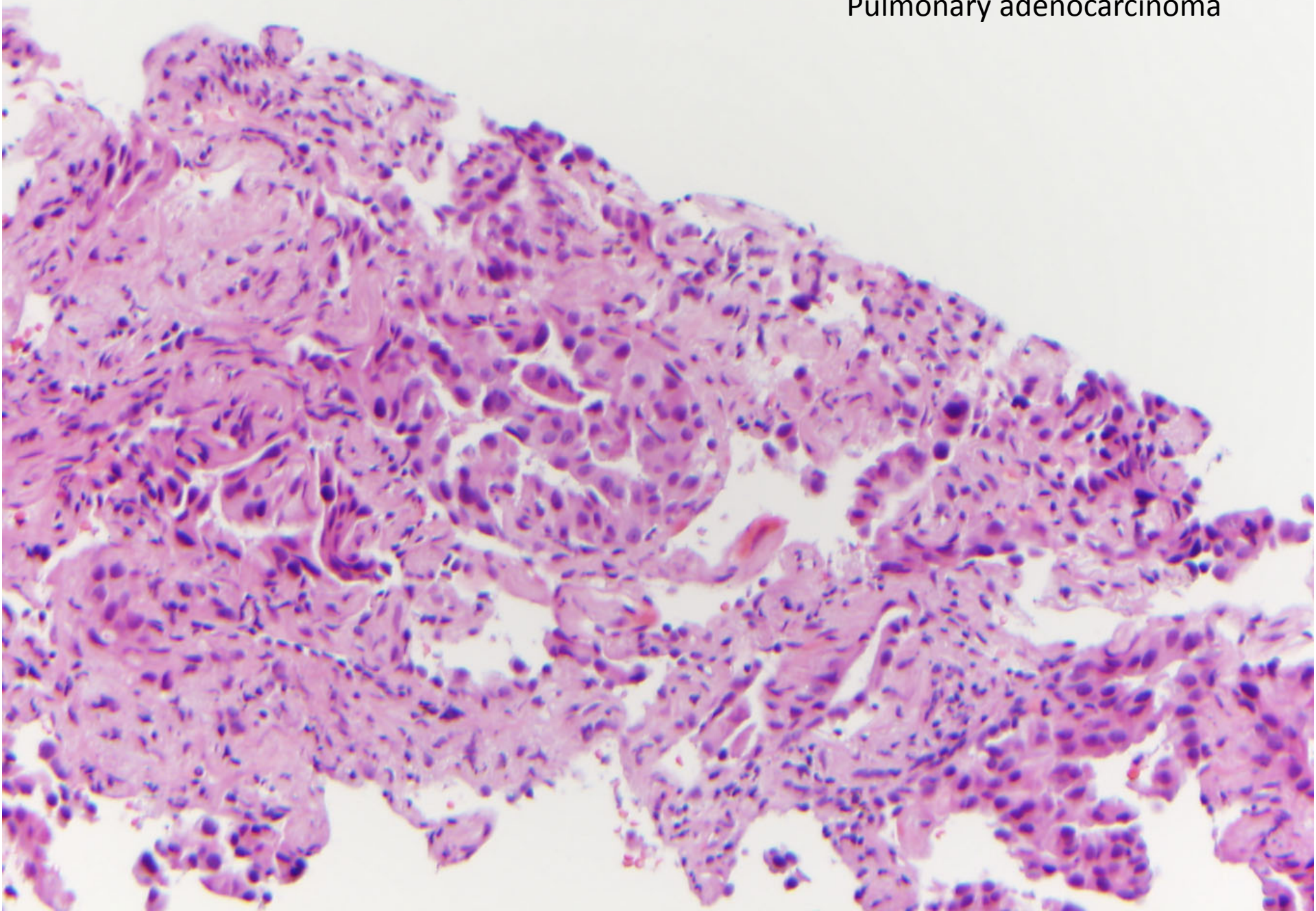






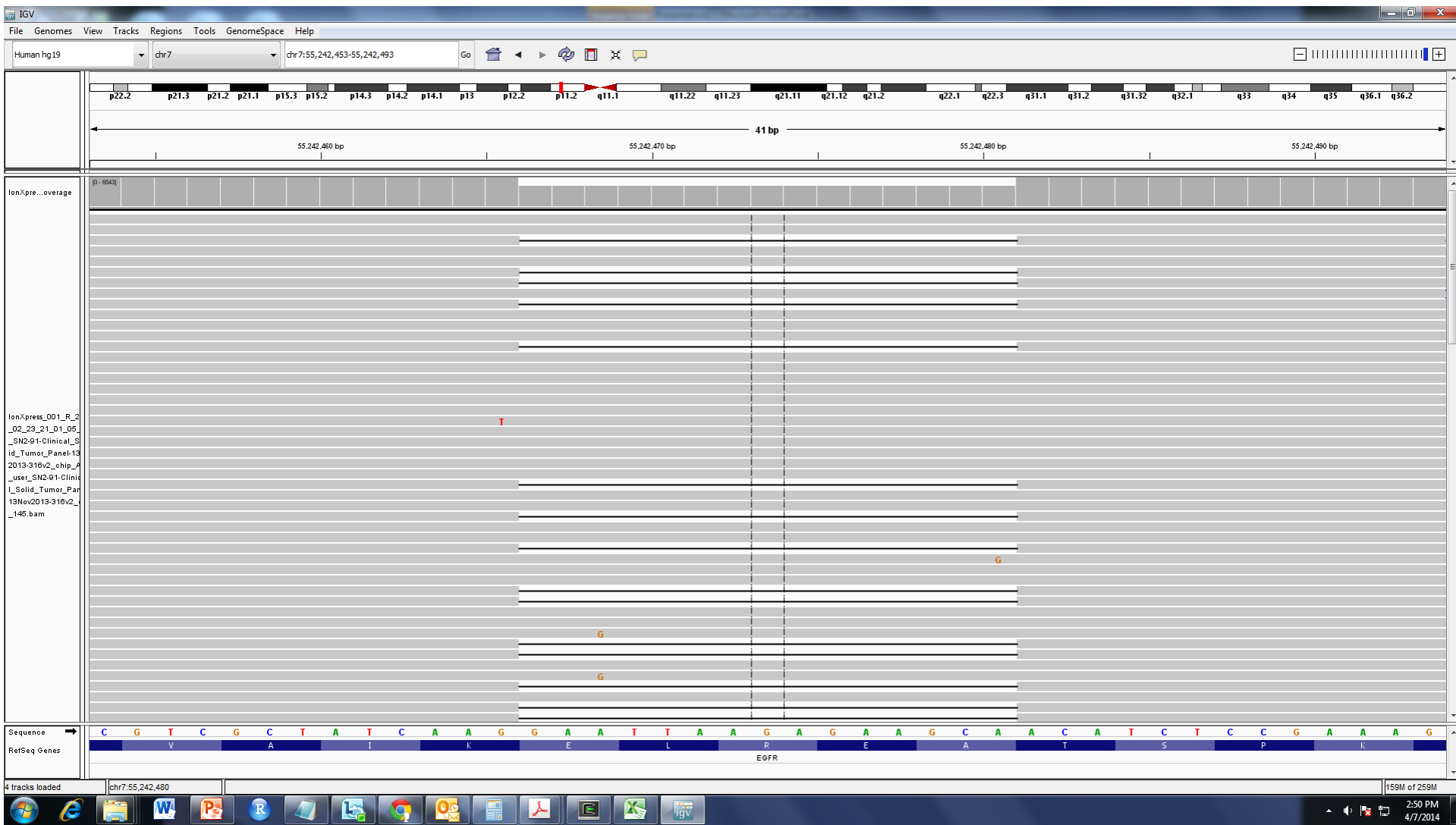


Pulmonary adenocarcinoma





# EGFR Exon 19: c.2236\_2250del, p.E746\_750del



# Case 4

- 73 year old woman with a right upper lobe lung mass. Bronchoscopic biopsy is performed. Tumor cells are positive for CK7, TTF1 and Napsin A, consistent with a primary lung tumor.
- EGFR mutation detection was requested
- EGFR exon 19 c.2236\_2250del, p.E746\_750del is detected.
- Patient is given erlotinib

# Summary

- Molecular diagnostics of solid tumors is a very important part of precision (personalized) medicine
- The number of types of tumors and relevant genes is rapidly expanding
- As the number of genes required to be evaluated increases, next generation sequencing will become increasingly attractive, as it will be cheaper and quicker

# Case 5

- 23 year old woman present with abdominal pain and hematochezia
- Colonoscopy reveals 20 adenomatous polyps
- Germline mutation testing for APC and MYH are negative
- Total colectomy and ileorectal anastomosis performed

# Case 6 continued

- 21 year old sister is scoped revealing a 4 cm cecal adenoma (with high grade dysplasia) and a 7 mm sigmoid adenoma
- Physical exam on first sister revealed axillary and inguinal freckling and two café au lait macules; second sister had one café au lait macule.
- Since APC and MYH wild type, considered Lynch syndrome

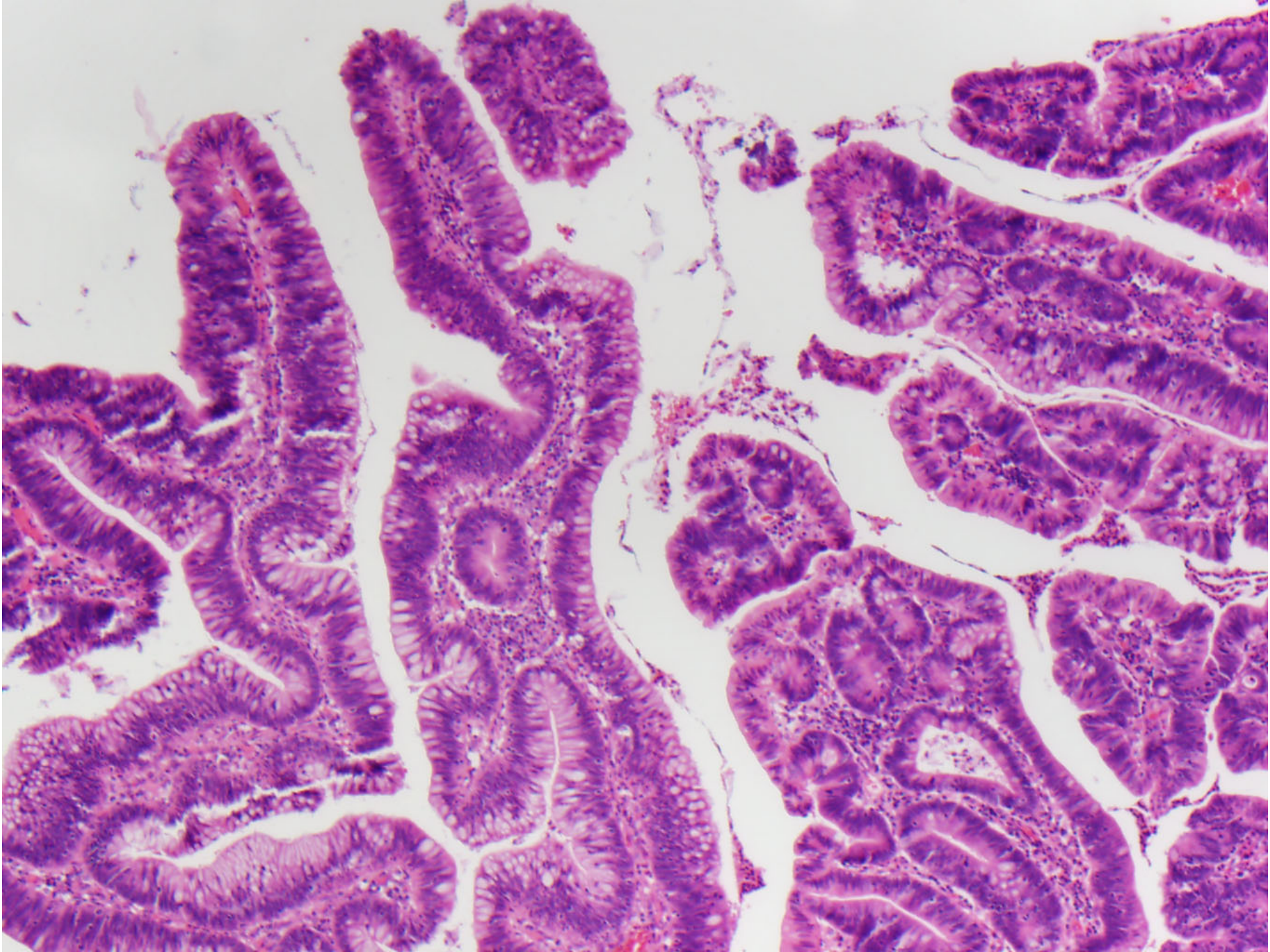


# What is Lynch syndrome?

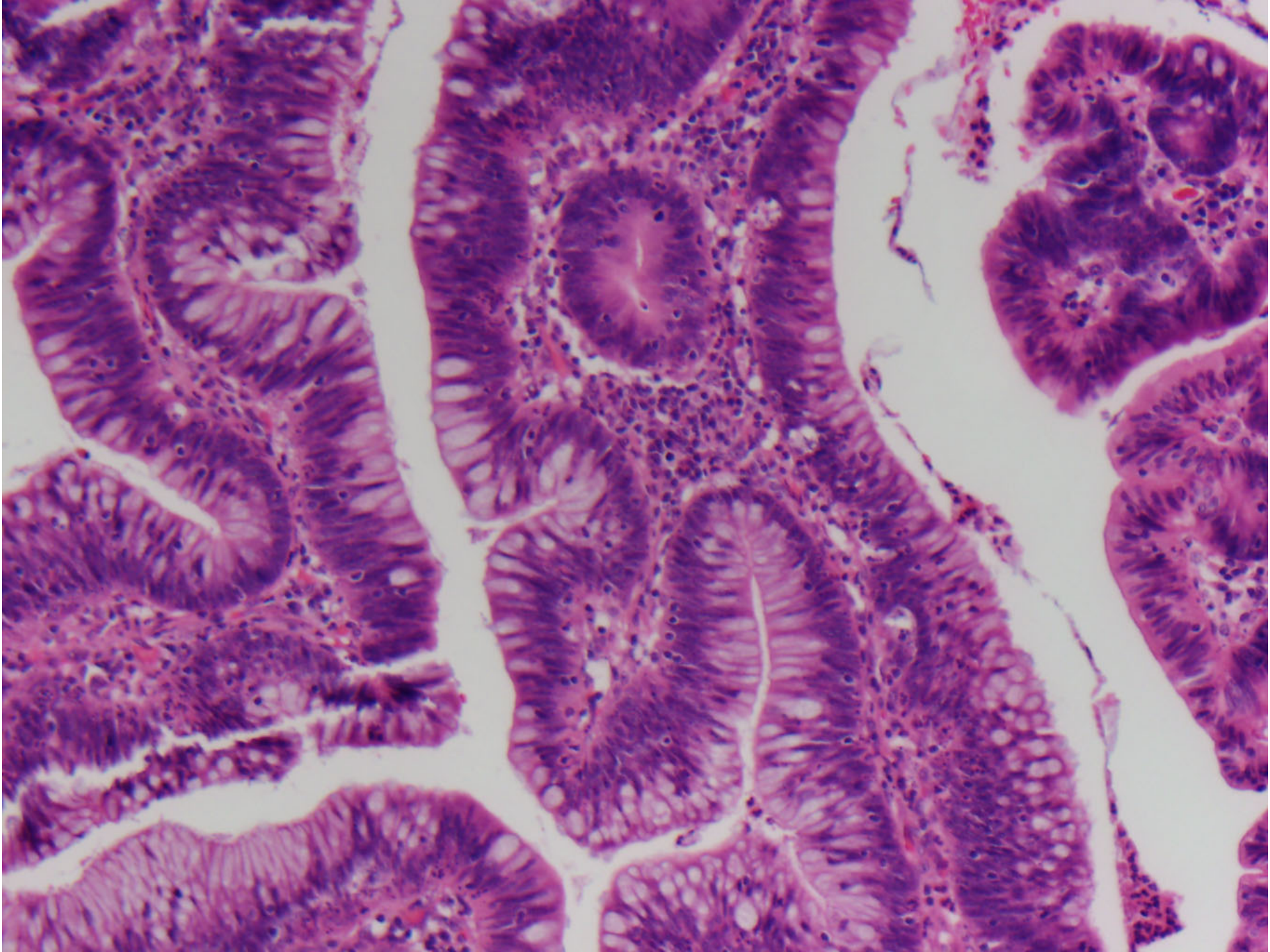
- Formerly HNPCC: hereditary non-polyposis colorectal cancer
- Young age onset right-sided colorectal cancer
- Also cancer of endometrium, ovary, renal pelvis, ureter, small intestine, stomach, hepatobiliary tract, pancreas
- May be difficult to recognize clinically or pathologically (unlike FAP, for example)
- Can use molecular features of tumor itself

# How do we work up Lynch syndrome?

- Determine if tumor is mismatch repair deficient
  - MMR antibodies (MLH1, MSH2, MSH6, PMS2)
  - Microsatellite instability by PCR
- Determine if mismatch repair deficient tumor is
  - sporadic: don't go on to germline testing
    - MLH1/PMS2 loss, MLH1 promoter methylation, BRAF mutation in colorectal cancer
  - possibly inherited: go on to germline testing

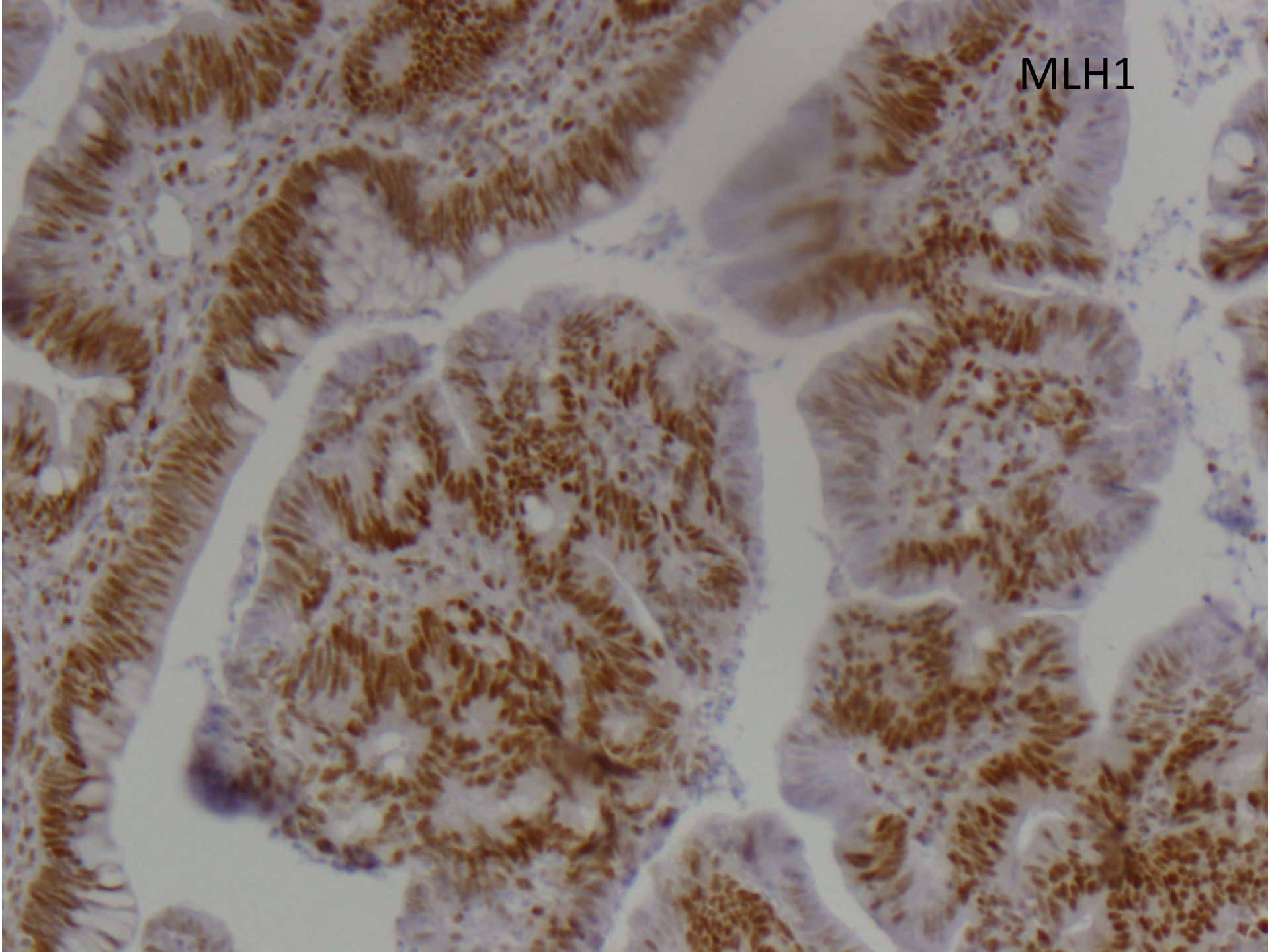






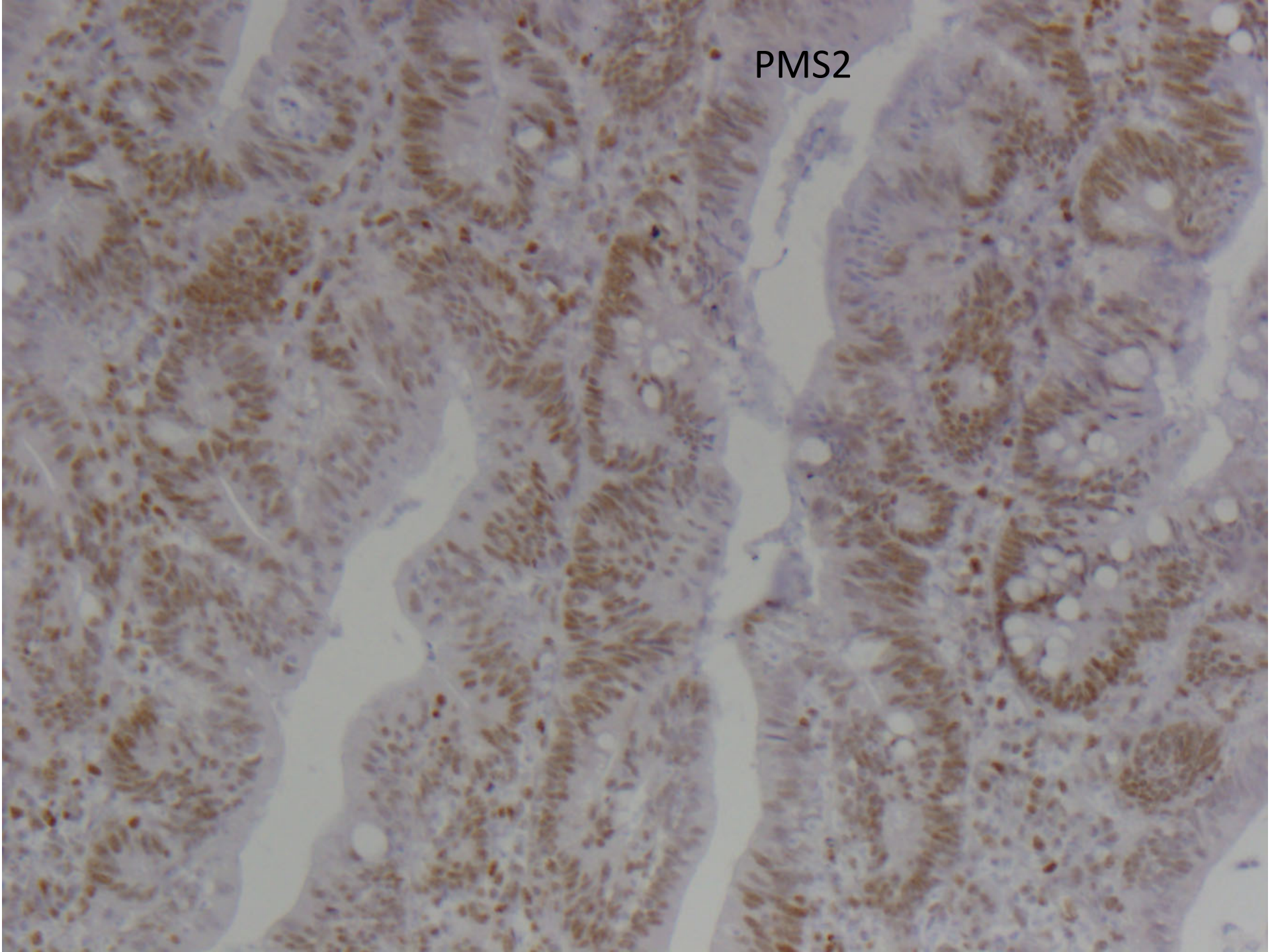


MLH1

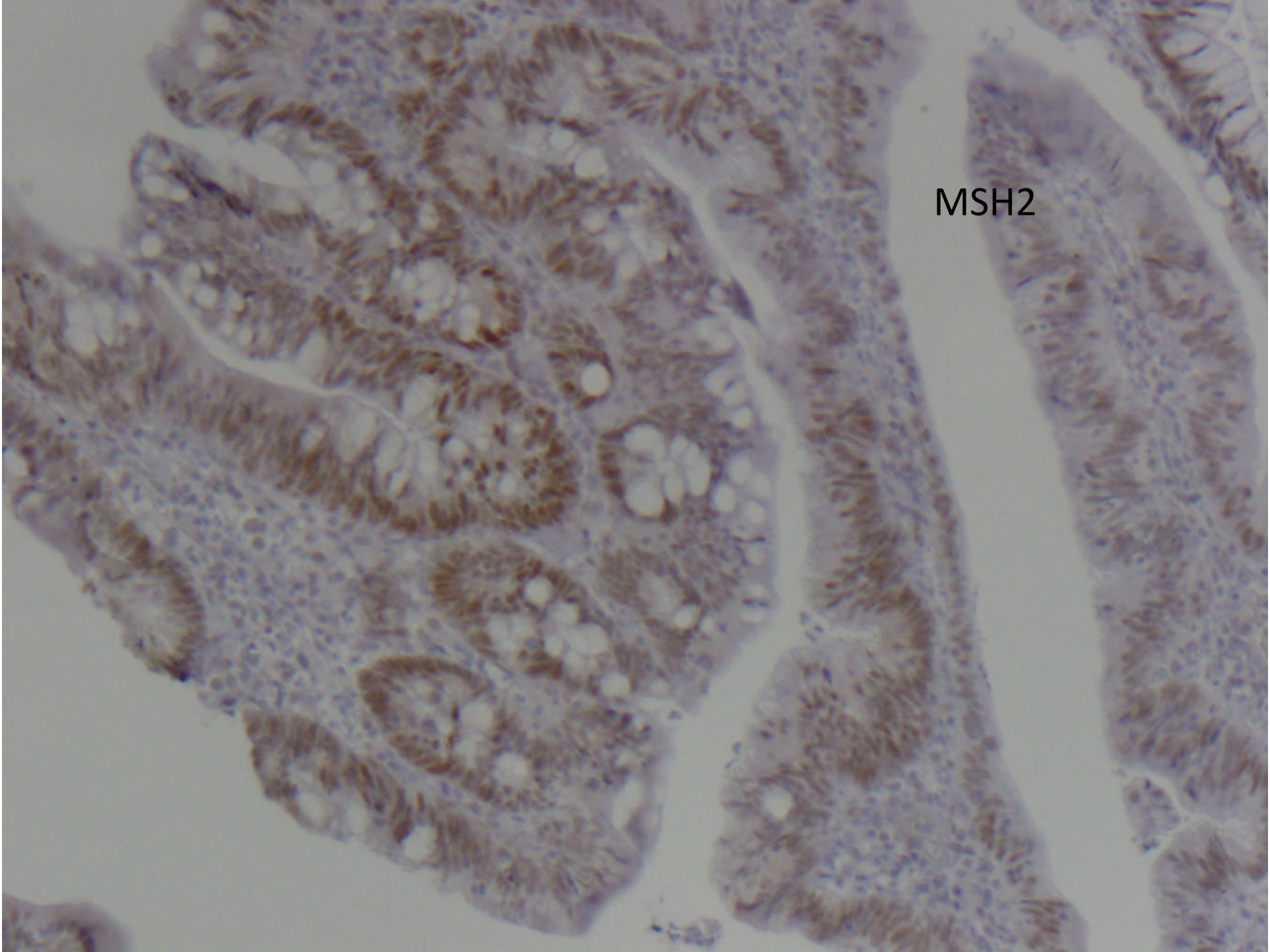




PMS2

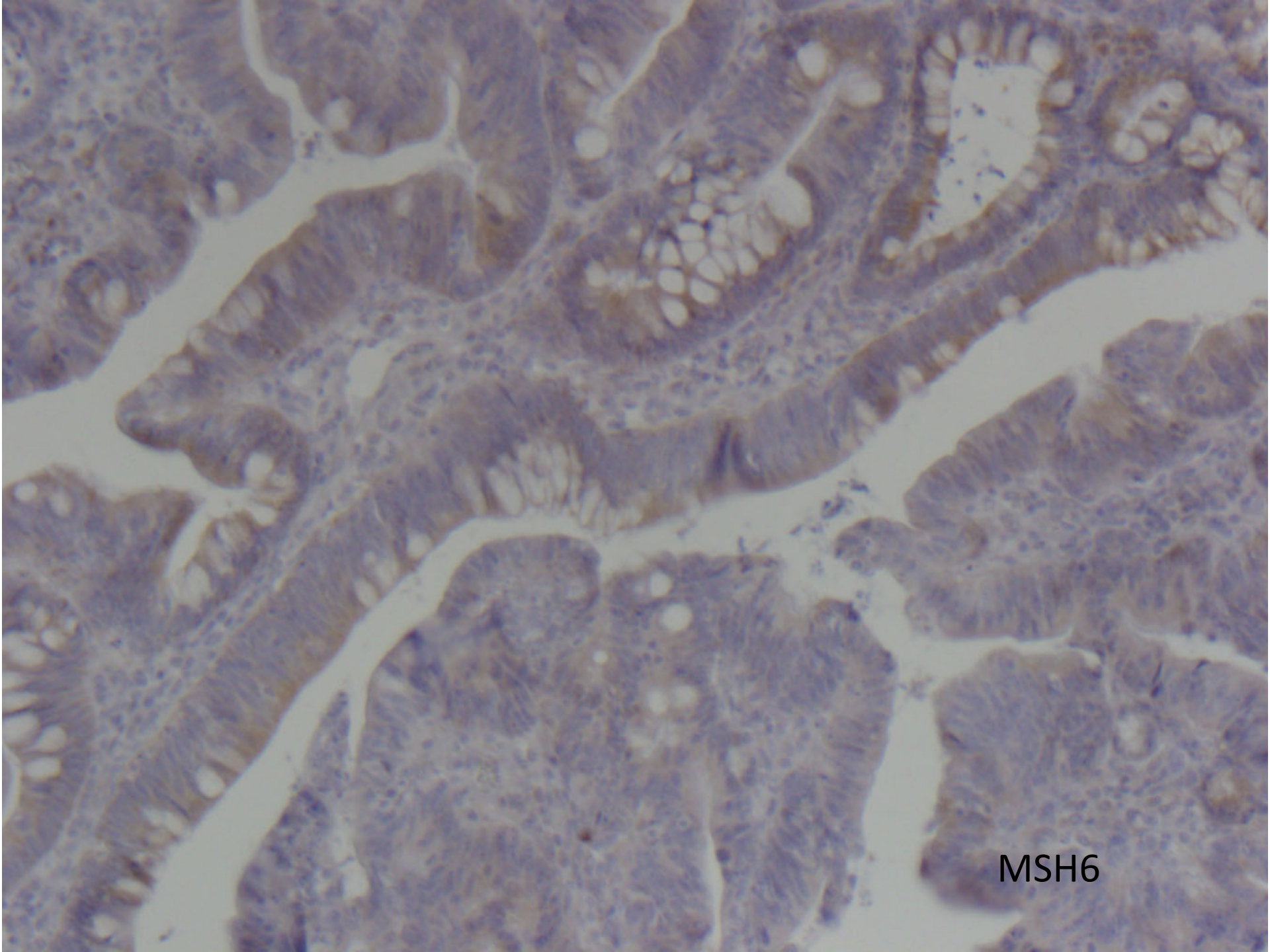






MSH2





MSH6

Sample File

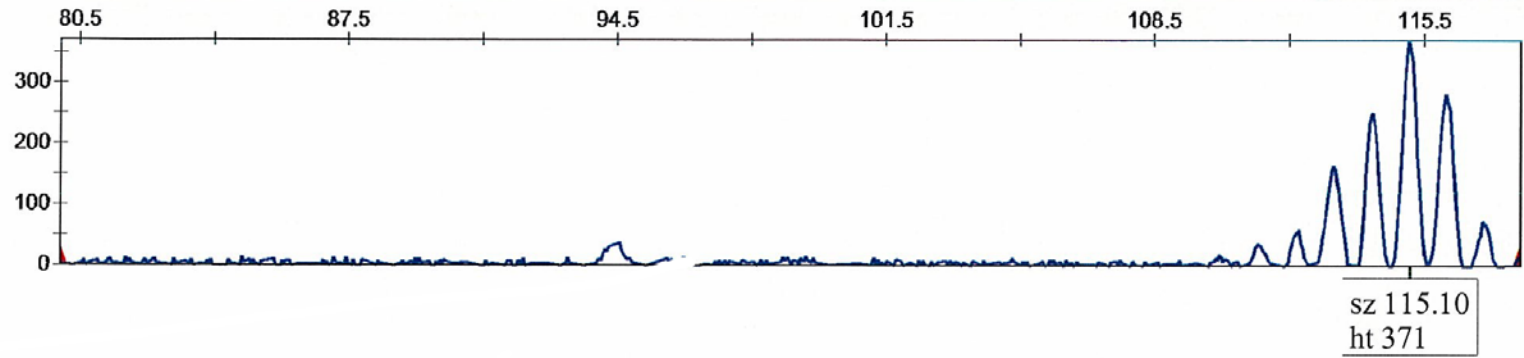
Panel

SQ

Promega MSI



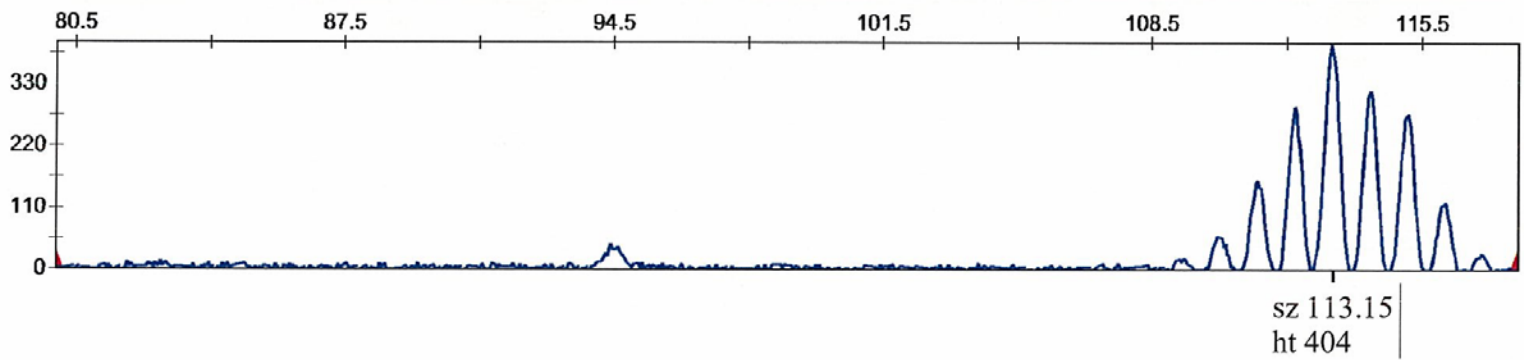
BAT-26



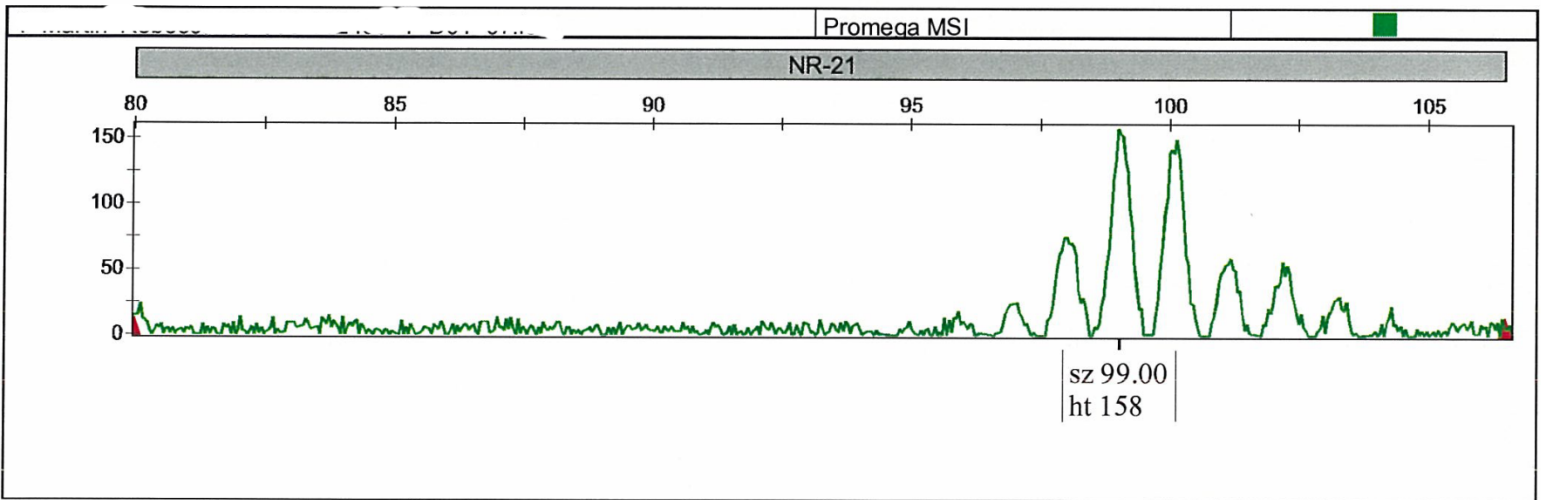
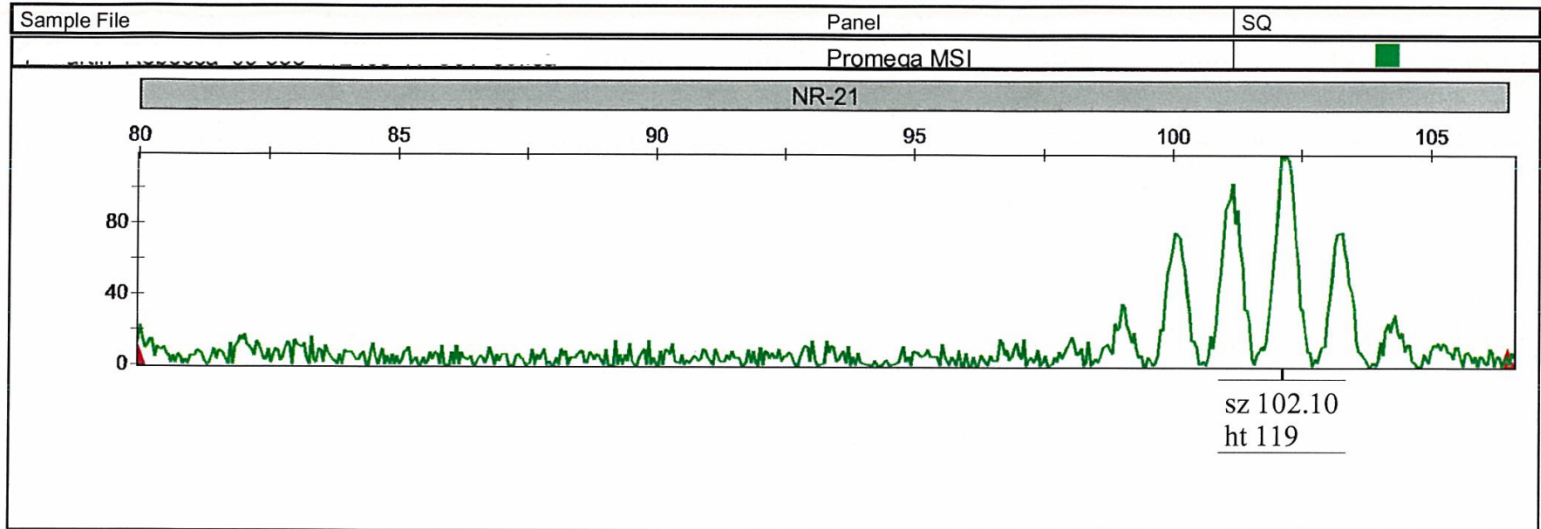
Promega MSI



BAT-26





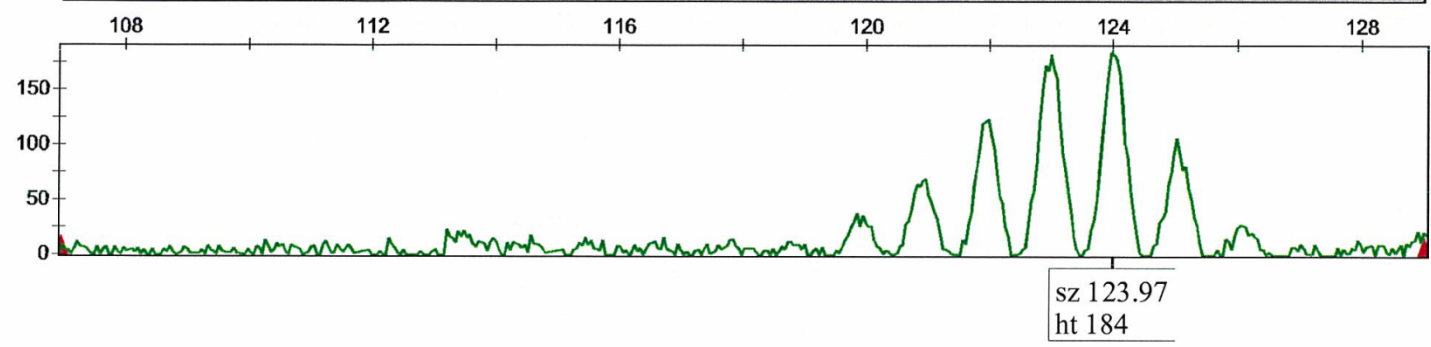


Sample File	Panel	SQ
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Promega MSI



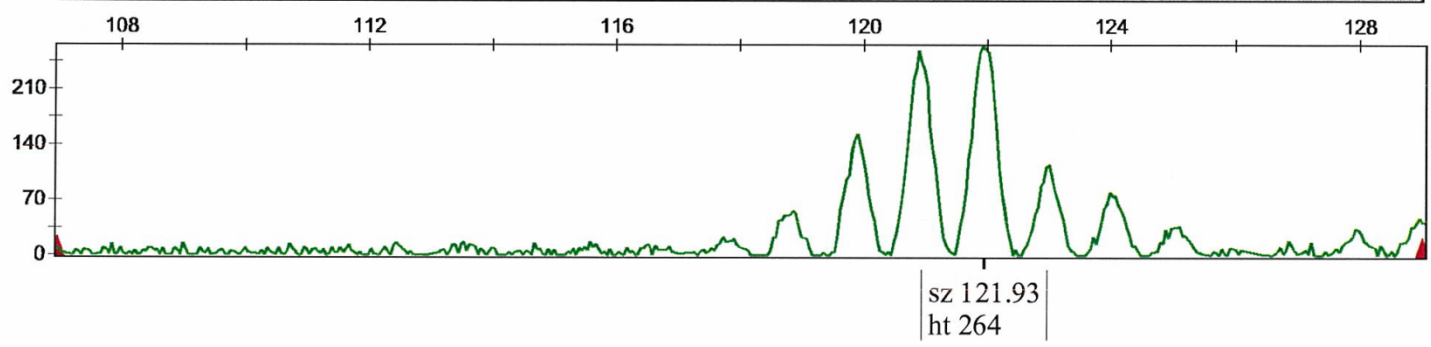
BAT-25

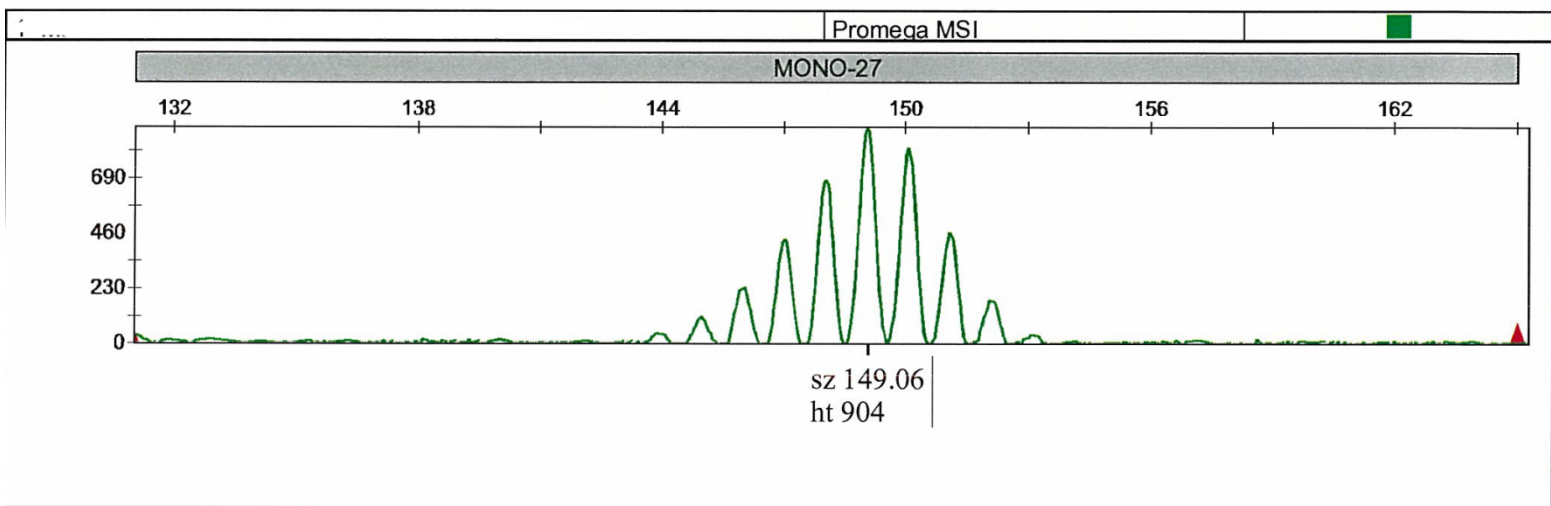
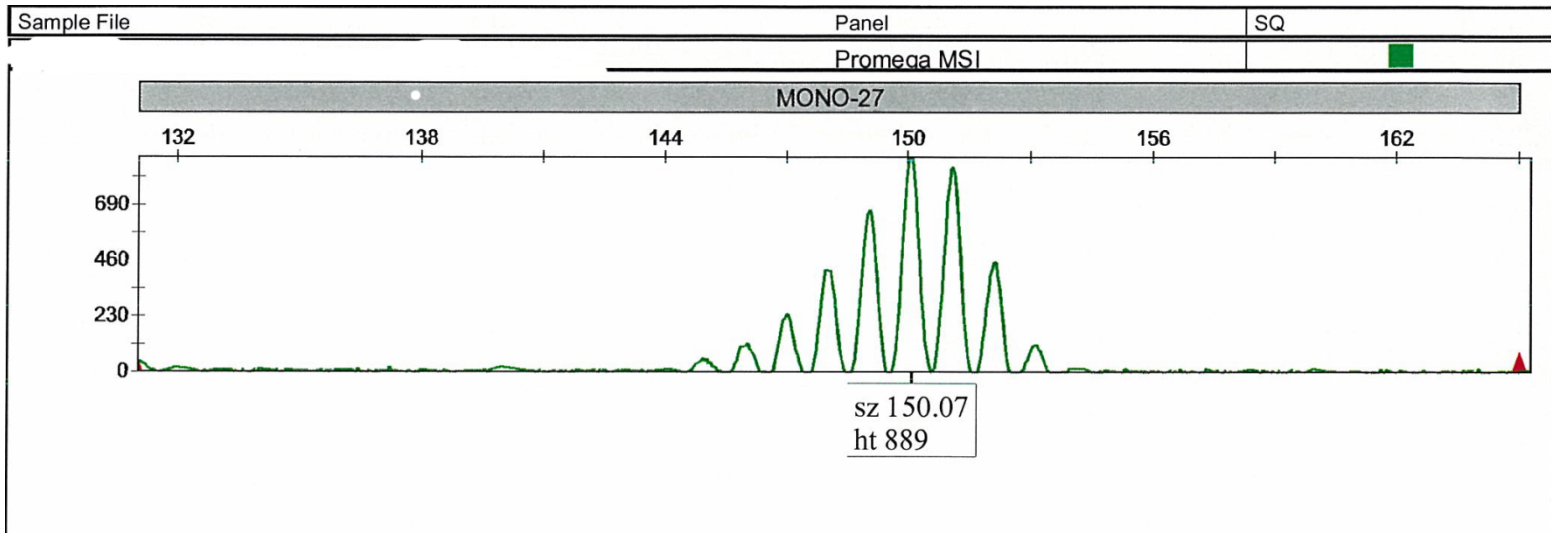


Promega MSI



BAT-25



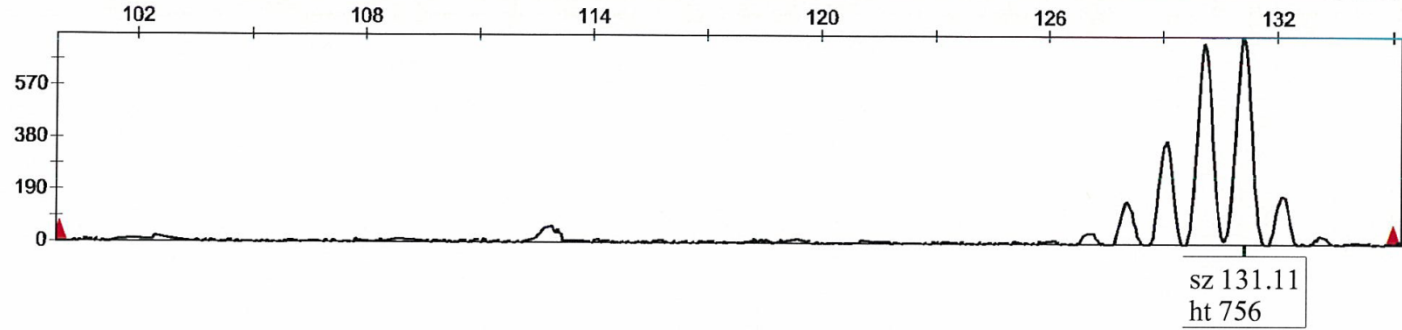


Sample File Panel SQ

Promega MSI



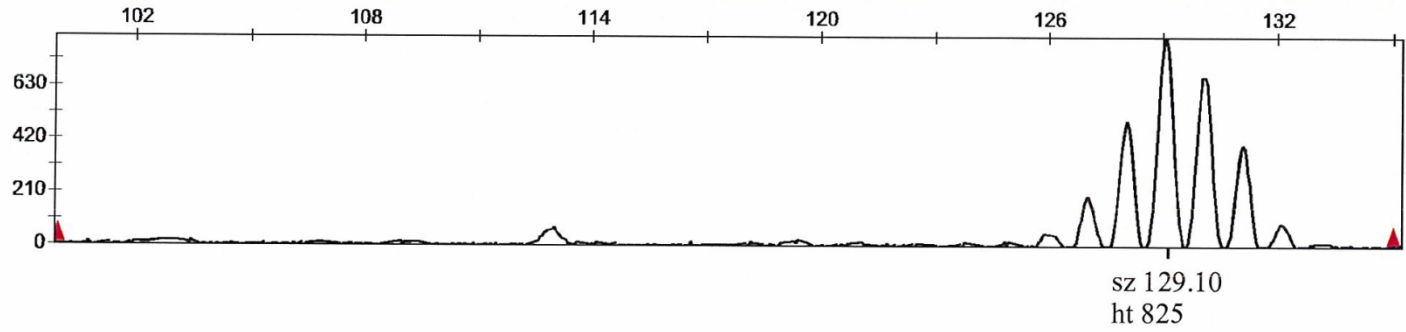
NR-24



Promega MSI



NR-24





- MMR IHC on the cecal adenoma:
  - normal MLH1, MSH2 and PMS2, uninterpretable MSH6 (tumor and internal control negative)
- MSI PCR was indeterminate (one of five mononucleotide repeats unstable)
- Germline MSH6 sequencing revealed bi-allelic mutations in both sisters

# Constitutional Mismatch Repair Deficiency Syndrome

- Bi-allelic mismatch repair gene mutations
- Childhood hematologic malignancies and brain tumors (mean age 5.5 and 8 yrs)
- Lynch syndrome-associated cancers, mean age 16 yrs (30 yrs earlier than average onset for Lynch syndrome)
- Almost all have NF1-like skin findings (café au lait macules and axillary/inguinal freckling)
- Consanguinity not uncommon

# Bi-allelic MMR mutations: molecular diagnostic difficulties

- Expect uninterpretable IHC, since both tumor and normal may lack protein expression
- PCR of brain tumors may not show MSI; MSI more likely to be seen in LS-associated tumors
- PCR of normal tissue does not show MSI
  - (? not enough replications)

# Constitutional Mismatch Repair Deficiency Syndrome

- PMS2 and MSH6 over-represented
  - Less penetrant, more viable in bi-allelic state
- Hematologic cancers more common in MLH1 and MSH2 bi-allelics, LS cancers more common in MSH6 and PMS2 bi-allelics (possibly due to increased survival to older age when LS tumors occur)
- Screening of heterozygous relatives may not have to be as stringent (less penetrant)
- At least some Turcot's may be bi-allelics



# Summary

- Can present as adenomatous polyposis (part of differential besides AFAP, MYH)
- Clues are NF1 skin features, childhood leukemias/lymphomas and brain tumors, LS-associated tumors
- Mismatch repair determination may be problematic

