ASCP 2021
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GIPS at ASCP
Tubular GI tract biomarkers: special considerations for cytology specimens

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Disclosures

- No relationships to disclose.

Table of Contents

introduction 1

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So Sunny! 5.

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how to make A Paper snowflak

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Objectives: Focus on Biomarkers HER2, PD-L1, Ki67

- Pre-analytical variables for cytology
- Analytical
  - Discuss the challenges in applying biopsy scoring systems to cytology
- Cytology-histology correlation
  - Review publications comparing biopsy and cytology
- Recognize gaps in knowledge of test performance

Biomarker testing: why cytology?

- Metastasis or recurrence with no prior biomarker testing
- Transfer of care, primary biopsy is not available
- Re-testing prior negative or scant results
- Small lesions with a difficult approach by interventional radiology
- Minimally invasive biopsy method
  - Effusions

Cytology specimen types: Exfoliative

Passive

- Pleural effusion
- Ascitic fluid

Instrumented

- Peritoneal washing
- Bronchial brushing/washing
- Esophageal brushing

Cytology specimen types: Fine needle aspiration

- For tubular GI, metastasis
- Guided by interventional radiology method
- Guided by endoscopic ultrasound
- Transcutaneous FNA of palpable lesions

Cytology preparations

*All can be used for molecular testing

Preparation*	Collection media/fixative	Notes for IHC/ISH
Air dried smear	None	Unstained can be used for ISH
Fixed smear	Alcohol (95% ethanol)	Variable, but often good results since Papanicolaou stain does not require de-staining
Cytospin	Variable	Immunohistochemistry, in situ hybridization
Liquid based (Thinprep, Surepath)	Methanol-based	Decreased staining intensity and some false negatives
Cell block	Variable - fresh, saline, RPMI or thrombin clots - 10% formalin - CytoRich red (alcohol) - liquid based or ethanol based with post-fix 10% formalin	Immunohistochemistry, in situ hybridization

Specimen type → Cytology collection media

	Received fresh, unfixed	Smear	Liquid based fixation
Small volume: - Esophageal brushing - Peritoneal washing	uncommon	+	+
Large volume: - Ascites - Pleural effusions - Drainage	+	+	variable
Fine needle aspiration	uncommon	+	+

Cytolyt® fixation for Thinprep preparation

- Altered expression of many markers
  - Absent/near absent: TTF-1, D2-40, CD20
  - Reduced: p16, p63, ER, S100, CD3, Calretinin, Chromogranin, Synaptophysin

Gruchy et al. Appl Immunohistochem Mole Morphol 2015.
Buonocore et al. Cancer Cytopathol 2019.

Summary of cytology pre-analytic variables

- Tumor sampling is most likely not the primary
  - Tumor heterogeneity may be a source of lack of concordance between
- Specimens may be fragmented and lack peritumoral tissue
  - Exfoliative
  - Fine needle (may have intact tissue fragments)
- Preparations may be manipulated further before testing
  - Destain/restaining steps for some smeared slides
- Fixation is variable and may have impact
  - Antigen retrieval
  - Unknown cold ischemia time

HER2 immunohistochemistry

- *HER2, HER2/neu, ERBB2* protooncogene encoding a tyrosine kinase receptor protein
- Amplification in 10-27% of gastric/GEJ adenocarcinoma
- Trastuzamab therapy for HER2 positive tumors defined by any FDA approved test
- AJCP/CAP/ASCO Guidelines 2016: FNA (Cell block) testing is an acceptable alternative to testing biopsy/resection

Bartley et al, AJCP 2016

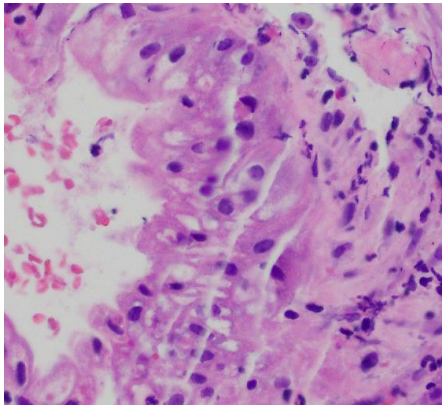
HER2 IHC on breast cytology

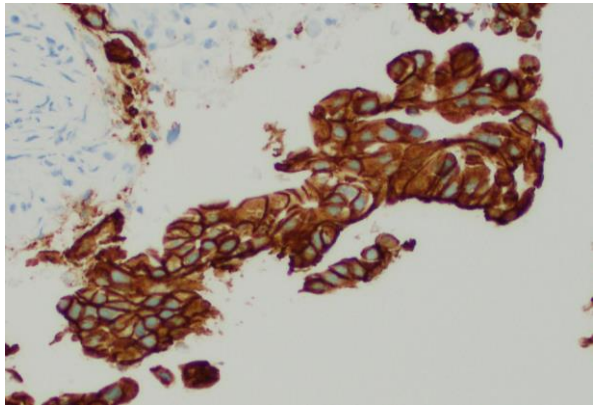
- There is functional equivalence for HER2 IHC between tissue and cell blocks if fixed in 10% formalin—*without ethanol or methanol fixation*
- Ethanol-fixed cell blocks have shown mixed/equivocal results for HER2

Vohra et al. Cancer cytopathology 2016
Bueno Angela et al, Cytopathology, 2013
Hanley et al. Cancer 2009
Gorman et al. Acta Cytol, 2012

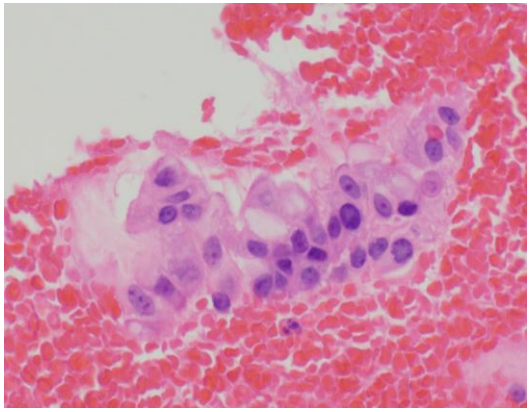
HER2 IHC

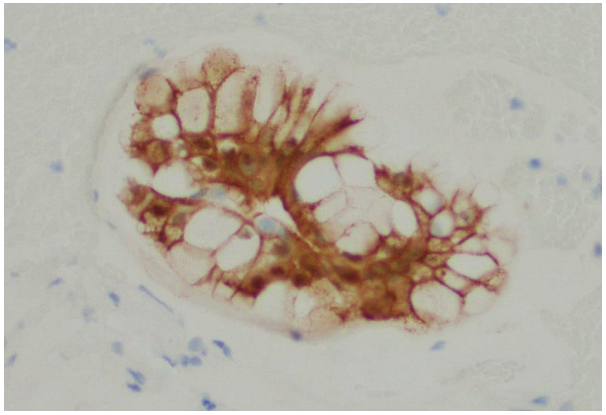
GEJ Biopsy





Lung FNA
Cell block
FFPE





Biopsy/cytology concordance for Gastric/GEJ adenocarcinoma

- Biopsy versus resection
  - Biopsy false negatives are possible
    - HER2 overexpression in 96% of surgical specimens vs 80% matched biopsy (Yan et al, J clin Pathol 2011)
  - Metastasis versus primary show mostly high concordance
    - 98.5% FISH concordance (biopsy or FNA) and 94.9% IHC concordance between primary and metastasis (Bozzetti et al, BJC, 2011)
    - 95% concordance for IHC (Wong et al, Pathology, 2015)
  - Tumor heterogeneity has led to preference for serial or repeat testing

HER2 Biomarker testing in effusions

- 40% of patients with gastric cancer have peritoneal disease leading to ascites
- Collecting ascitic fluid is minimally invasive
- Testing of this fluid characterizes the actively spreading disease clone
- Clinician requests are not uncommon!

Martin et al, Mayo Clin Proc. 1986

HER2 testing on effusions: methods

	Wong et al. Diagnostic Cytopathology, 2014	Kim et al. BMC Cancer 2015
Study size, paired primary samples	N=46, 18 paired	N=45, all paired
Fixation	Fresh > thrombin clot > formalin	Ethanol fixed
Antibody	Dako pc CERB2	Ventana 2B5
Positive HER2 criteria (Hofman criteria)	2+/3+ IHC and SISH amp or 3+ IHC only	IHC3+ or SISH amp

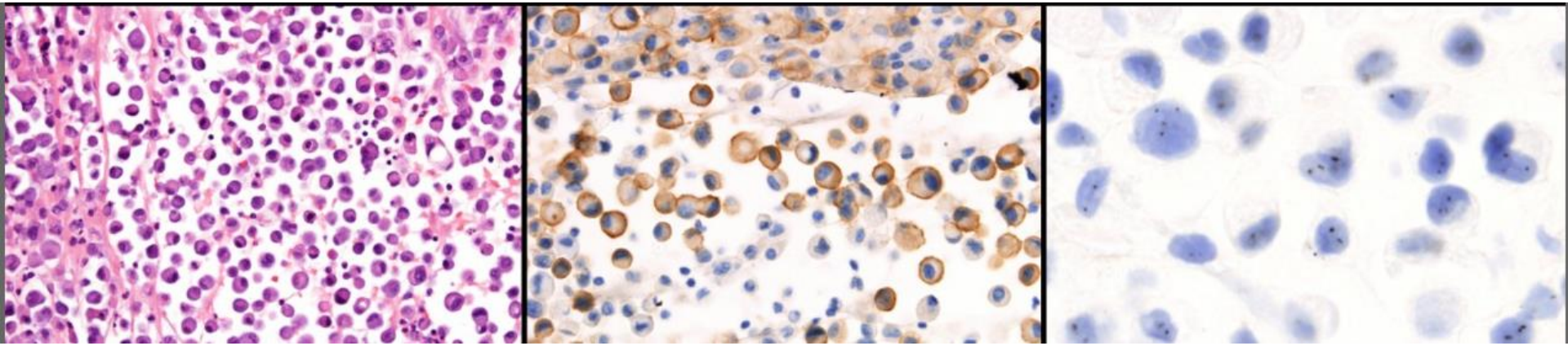
Cold ischemic time? Formalin fixation 6-70 hours?

HER2 testing on effusions: results

	Wong et al. Diagnostic Cytopathology, 2014	Kim et al. BMC Cancer 2015
Study size, paired primary samples	N=46, 18 paired	N=45, all paired
Cellularity	24% had low tumor content	Half had low tumor content
% HER2 cytology positive	7%	6.7%
IHC-SISH correlation	Only IHC 3+ were SISH amp (33 cases tested) One IHC3+ was SISH non-amp	Only IHC 3+ were SISH amp (10 cases tested)
Serial testing	Not done	Serially tested fluids had inconsistent HER2 results
HER2 IHC concordance between primary and cytology	100% concordance for HER2 primary and cytology	No concordance for HER2 between primary and cytology

Practical observations: HER2 IHC in effusions

- Low rate (7%) effusion HER2+ compared to 10-30% prevalence of gene amplification
  - Testing effusions has over representation of diffuse cancers and they have lower prevalence of HER2 amp/overexp
    - e.g. Wong et al's primary cancer types: 43% diffuse, 38% mixed intestinal/diffuse type, 19% intestinal
- Need improved criteria for dispersed cells
  - Wong et al: 65% cases had no tumor cell clusters present
- In both studies only IHC3+ corresponded to amplification
  - How do we report a specimen with convincing 3+ labeling but <5 cells?
- Tumor heterogeneity may lead to discordance
  - Kim et al. showed no concordance with primary and discordance on serially tested fluids



Single tumor cells

Compressed cytoplasm,
Mimics membranous staining

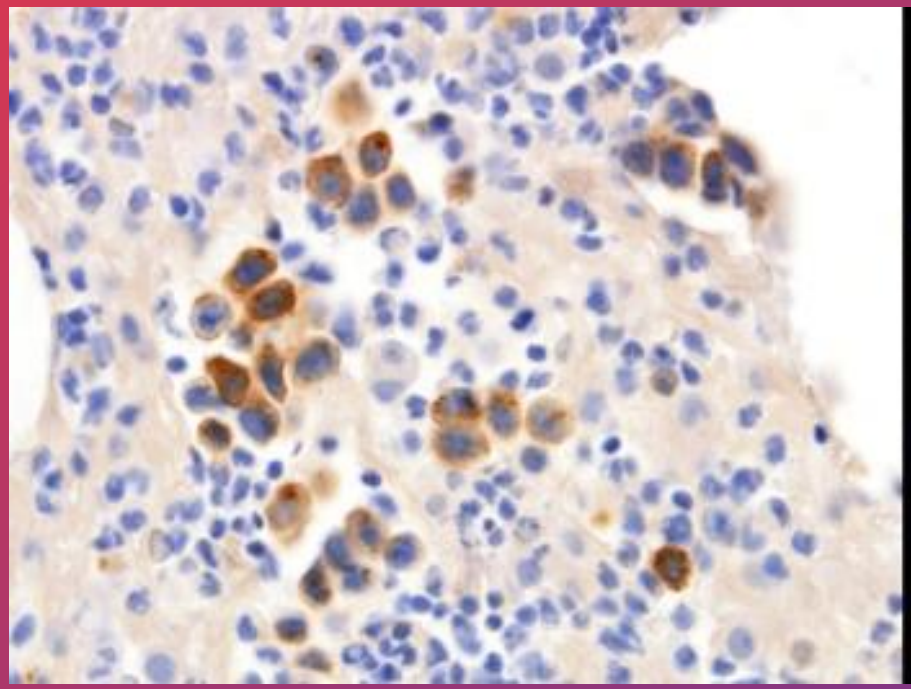
Absence of HER2 gene amp
By SISH

Wong et al. Diag Cytopath, 2014.

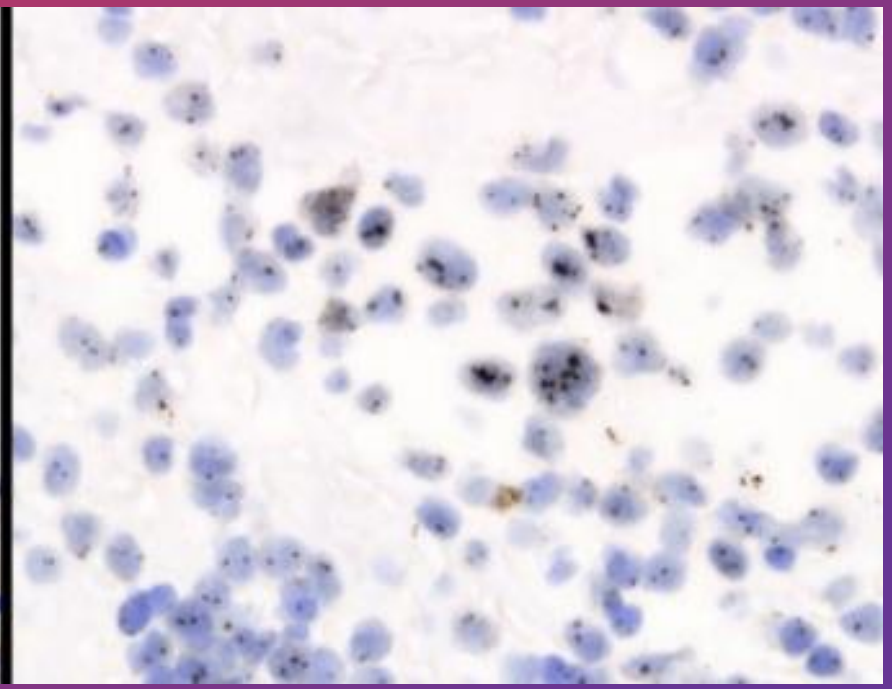
IHC was “more granular” than the linear pattern seen in histology

“microvillous” transformation on cells in effusion

? altered antigenicity



“Granular” membraneous
And cytoplasmic staining



Amplification of HER2 gene
By SISH

Wong et al. Diag Cytopath, 2014.

Practical observations: SISH in effusions

- Non-pleomorphic neoplastic single cells in fluid are difficult to distinguish from mesothelial cells
  - Requires close correlation between IHC and SISH
    - difficult
    - time consuming
    - cytomorphology expertise is necessary

Wong et al. Diag Cytopath, 2014.

PD-L1 IHC: background

- Multiple antibody clones
- Commercially available IHC assays are validated with FFPE
- Site specific reporting
  - Tumor proportion score (TPS) (lung)
  - Combined positive score (CPS) (upper GI, head and neck)
- **Very limited reporting on cytology experience**
  - Mainly data on NSCLC

PD-L1 cytology testing: NSCLC

- Tumor proportion score (TPS) only
- Minimum 100 well-preserved cells recommended
- High concordance with biopsy and resection (clones 22C3, SP263)
- Several studies show type of fixative did not matter if properly optimized and validated*
- Cytology PDL1 has intra-observer variability…
- Cytology PDL1 is reliable for NSCLC

*IASCL requires validation and QC measures for cytology.

Reviews:
Iaccarino et al, Cytopathology 2021.
Tejerina E et al, Front Med 2021.
Gosney et al. Lung Cancer 2020.
Mansour et al. Acta Cytol 2021.

PD-L1 cytology testing: practical points

From NSCLC experience, yet relevant to testing other tumors:

- Evaluate well preserved, non-overlapping cells
- Must distinguish tumor from macrophages
- 3D intact cells have strong staining (membrane is intact)
- True membranous staining is positive
  - Weak cytoplasmic staining is not positive
  - Peri-nuclear dot like staining with 22C3 assay may be seen

Iaccarino et al. Cytopathology 2021.
Tejerino et al. Front Med. 2021.

PD-L1: the CPS score on FNA cytology

- Head and neck cancer study
- 10 LN FNA cases in a cohort of 20
- Combined positive score (CPS) method on cell block
  - Only inflammatory cells in same tissue fragment as tumor cells are counted
  - Exclude inflammatory cells in necrosis or normal lymphoid tissue
- Positive threshold ≥1% had 70% accuracy
  - Low negative predictive value (28%)
    - False negative example: the prominent tumor immune response seen in primary was lacking in small biopsy and tumor cells were negative
    - Suggest re-test negatives

Paintal et al. Head Neck Pathol. 2020.

Summary: a knowledge gap exists for feasibility of PD-L1 CPS scoring on cytology for GEJ cancer

- Reliable immunohistochemical stain if optimized and validated on cytology
  - Suggest at least 100 cells; more cells is better
- Inadequate sampling of tumor associated stroma/inflammation may lead to false negative CPS
- TPS score is not relevant for GEJ/gastric cancers
  - Informally, estimated half of CPS >1% is due to inflammation
  - Unacceptably low negative predictive value projected
  - Negative result is non-informative and may be misleading to clinician
  - Exfoliative specimens (Ascitic fluid, washes) are not appropriate for testing

Ki67 grading on cytology

- For tubular GI specimens, Ki67 is a biomarker for grading neuroendocrine neoplasms
- Evaluation of Ki67 performance on cytology specimens has mainly been done on pancreatic neuroendocrine neoplasms

Ki67 grading NET on cytology

- For NET recurrence, an increase in grade correlates with decrease in progression free and overall survival

- Core and cytology grading have prognostic value and are nearly equivalent.
  - Both can underestimate grade due to these factors:
    - Sampling (core, cytology)
    - Fixation (cytology)

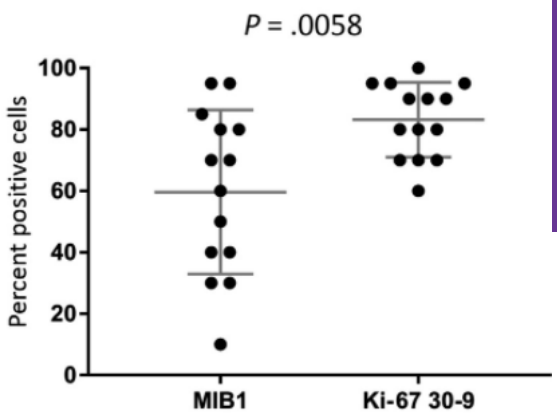
Sadot et al. Ann Surg Oncol, 2016.
Keck et al. Ann Surg Oncol, 2017.

Cancer Cytopathology

Original Article | Free Access

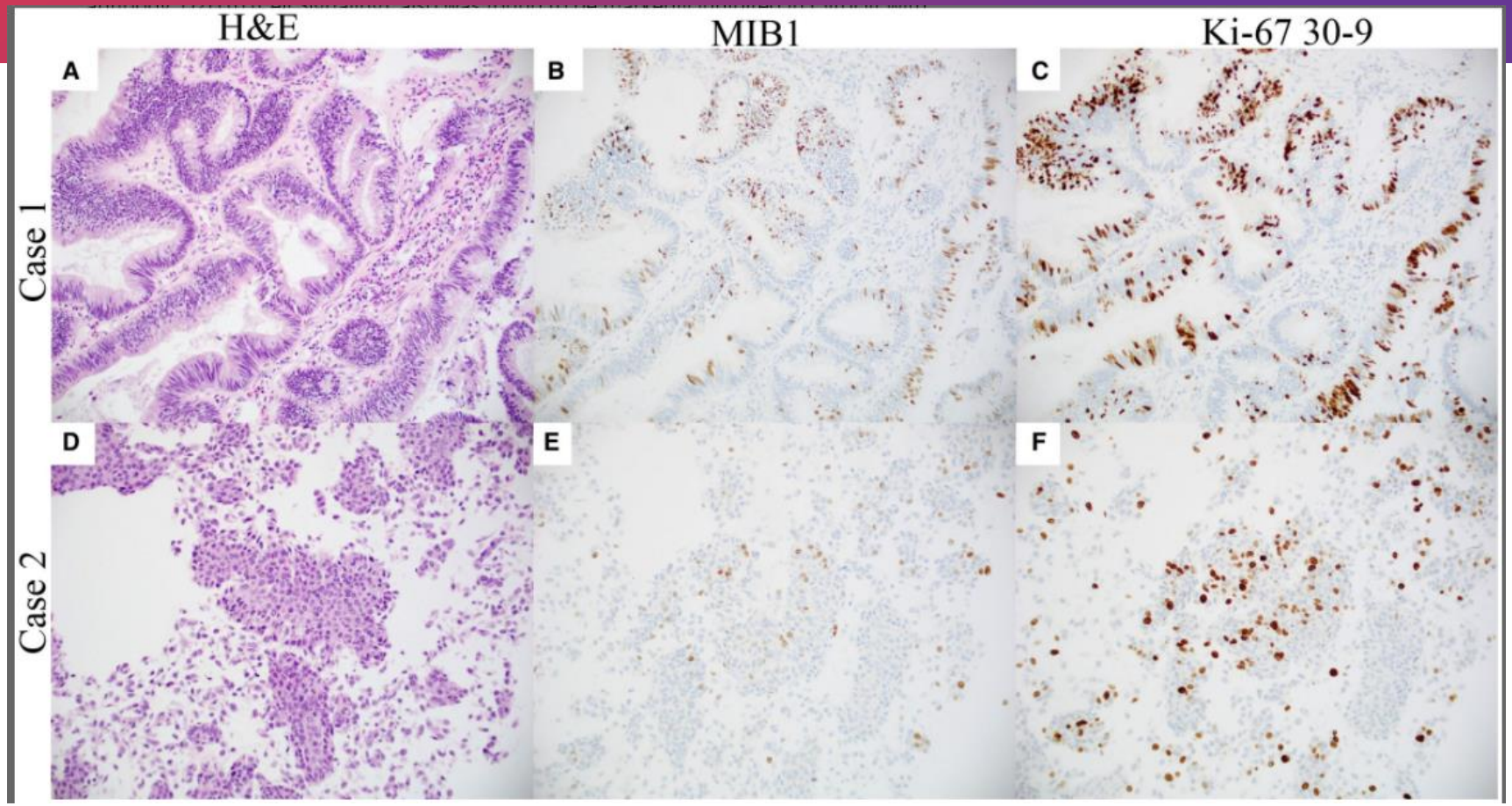
CytoLyt fixation significantly inhibits MIB1 immunoreactivity whereas alternative Ki-67 clone 30-9 is not susceptible to the inhibition: Critical diagnostic implications

Darren J. Buonocore MD, Fumiko Konno MD, Achim A. Jungbluth MD, PhD, Denise Frosina BS, Mariam Fayad BS, Marcia Edelweiss MD, Oscar Lin MD, PhD, Natasha Rekhtman MD, PhD



- Specimens placed directly into Cytolyt media at collection vs formalin
- Compared performance of MIB1 (DAKO/Agilent), Ki-67 30-9 (Ventana), D2H10 (cell signaling) antibody
  - MIB1 and D2H10 showed lower % positive with lower staining intensity
- Post-fixation in formalin does not result in restoration of immunoreactivity

Cytolyt-fixed

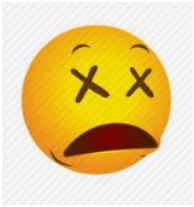


Buonocore et al. Cancer cyto 2019.

How do we grade NET on cytology?

- We can stain smears or cell blocks
- Must validate antibody for cytology specimens
- Count low and high intensity staining in non-overlapping cells

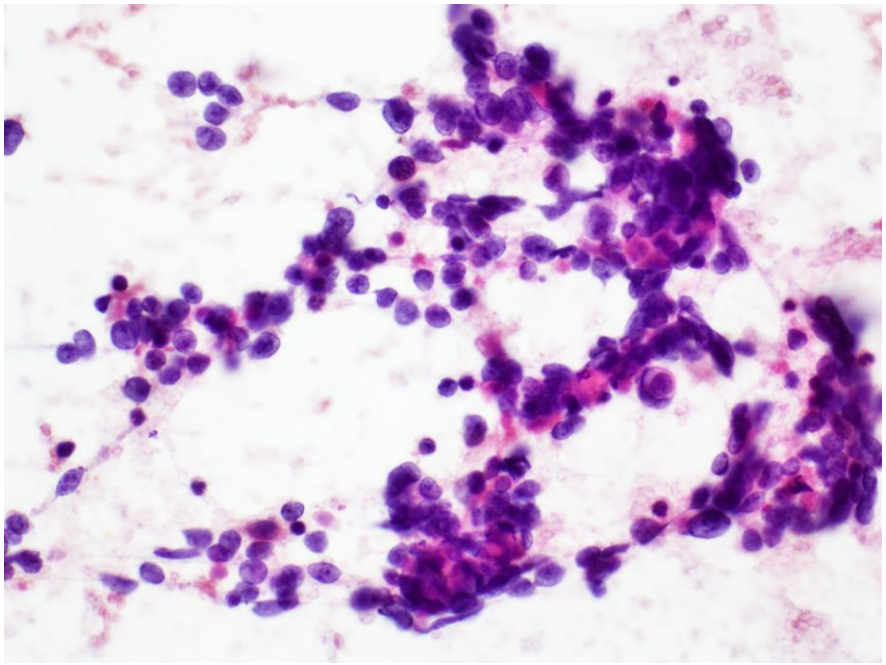
- Manual counting is best
  - No “eyeballing!”

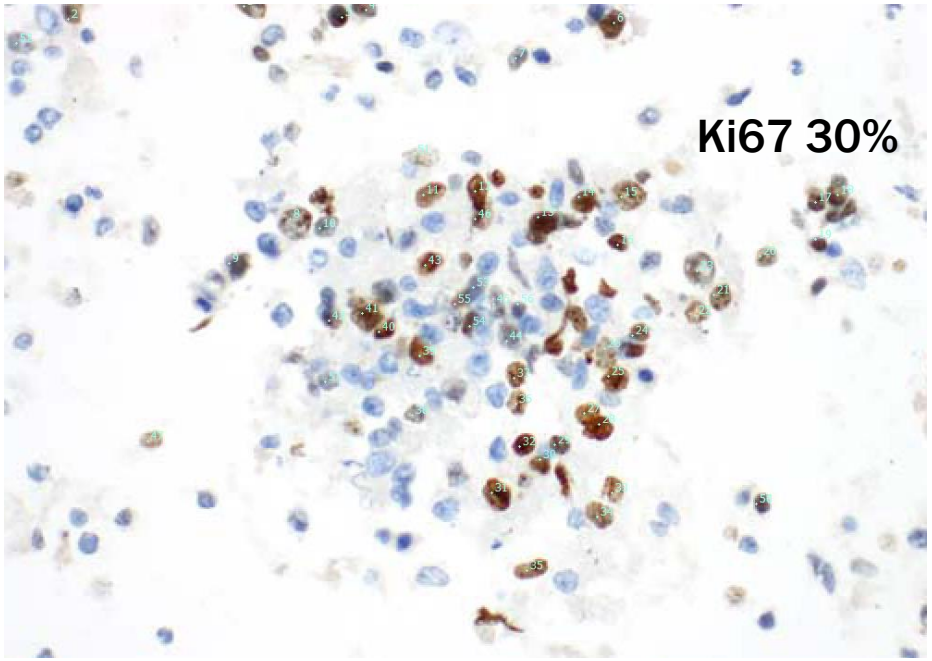


Sigel et al. Cancer Cyto, 2017.
Farrell et al. Cancer Cyto, 2014.
Tang et al. AJSP, 2012

Manual counting for Ki67 proliferation index: cytology

- Count 500-2000 cells (cell block, direct smear)
- Report a calculated value for Ki-67 index, not an estimate or range such as “<5%”
- Cytopathology expertise is essential! Do not count lymphocytes, histiocytes, and endothelial cells





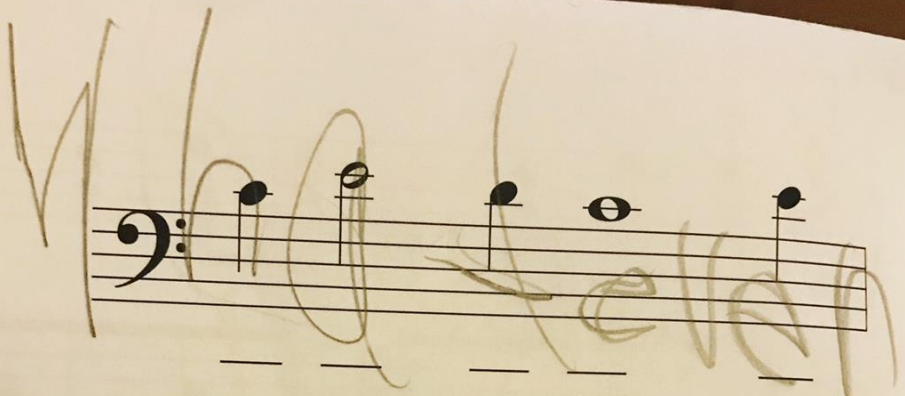
Boutsen et al, Neuroendocrinology, 2018

Key points: GI Biomarker testing on cytology

Published experience is limited and most information is extrapolated from other tumor sites…but it can be done!

Across labs there is wide variation!

Validation studies must address pre-analytic factors to support cytology preparations



- Cover up the notes to the left.
  Quiz yourself and name these notes.

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nes aloud.

Whirling Leaves

____ 5-Finger Scale

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Validation of a Cytotechnologist Manual Counting Service for the Ki67 Index in Neuroendocrine Tumors of the Pancreas and Gastrointestinal Tract

Jennielee Cottenden, MD; Emily R. Filter, MD, FRCPC; Jon Cottreau, MD; David Moore, MMedSc; Martin Bullock, MD, FRCPC; Weei-Yuarn Huang, PhD, MD, FRCPC; Thomas Arnason, MD, FRCPC

• ***Context.***—**Pathologists routinely assess Ki67 immunohistochemistry to grade gastrointestinal and pancreatic neuroendocrine tumors. Unfortunately, manual counts of the Ki67 index are very time consuming and eyeball estimation has been criticized as unreliable. Manual Ki67 counts performed by cytotechnologists could potentially save pathologist time and improve accuracy.**

Objective.—**To assess the concordance between manual Ki67 index counts performed by cytotechnologists versus eyeball estimates and manual Ki67 counts by pathologists.**

Design.—**One Ki67 immunohistochemical stain was retrieved from each of 18 archived gastrointestinal or pancreatic neuroendocrine tumor resections. We compared pathologists' Ki67 eyeball estimates on glass slides and printed color images with manual counts performed by 3 cytotechnologists and gold standard manual Ki67 index counts by 3 pathologists.**

Results.—**Tumor grade agreement between pathologist image eyeball estimate and gold standard pathologist manual count was fair ($\kappa = 0.31$; 95% CI, 0.030–0.60). In 9 of 20 cases (45%), the mean pathologist eyeball estimate was 1 grade higher than the mean pathologist manual count. There was almost perfect agreement in classifying tumor grade between the mean cytotechnologist manual count and the mean pathologist manual count ($\kappa = 0.910$; 95% CI, 0.697–1.00). In 20 cases, there was only 1 grade disagreement between the 2 methods. Eyeball estimation by pathologists required less than 1 minute, whereas manual counts by pathologists required a mean of 17 minutes per case.**

Conclusions.—**Eyeball estimation of the Ki67 index has a high rate of tumor grade misclassification compared with manual counting. Cytotechnologist manual counts are accurate and save pathologist time.**

(*Arch Pathol Lab Med.* 2018;142:402–407; doi: 10.5858/arpa.2017-0203-OA)

Neuroendocrine tumors (NETs) of the gastrointestinal tract and pancreas are uncommon neoplasms with marked heterogeneity in clinical behavior.[1,2] Ki67 immunohistochemistry has emerged as an important prognostic

the Ki67 immunostain in a hot-spot microscopic field containing at least 500 to 2000 tumor cells.[4] Although time consuming, a pathologist manual count of the Ki67 index using a printed photomicrograph is increasingly considered