Prepared by Dr. Kurt Schaberg

Immunohistochemistry

Fundamentals to Consider

1) Use it to *support* your morphologic/clinical impression

Immunohistochemistry (IHC) is an adjunctive tool to *help* you *support* your diagnosis or offer predictive/prognostic information (not make the diagnosis for you! ;-). The results must be interpreted in their morphologic and clinical context. Always be wary of making too much of a diagnosis based solely on a stain—the morphology and clinical scenario should match too!

2) It's a matter of *probability*, with nothing being absolute

Staining, particularly when it comes to lineage, site, or differentiation, is essentially a matter of statistics. While *most* classic lung adenocarcinomas may stain with TTF1, many do not. Staining is best used to support or argue against a diagnosis—it seldom *proves* anything. Know the sensitivity and specificity for each specific clinical context, otherwise you're just playing the lottery.

3) Will you say the same thing regardless?

If you're going to say the same thing regardless of a staining result—consider *not* doing the stain. It's just going to add cost and potentially a conflicting result you'll have to explain away!.

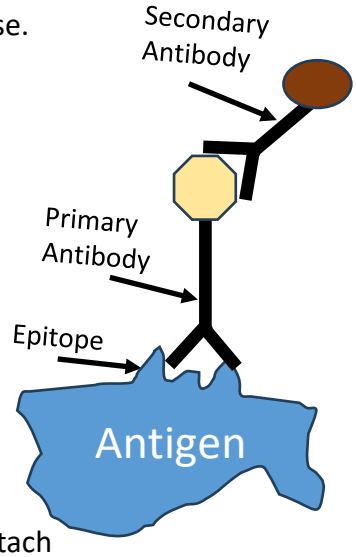
How IHC Works

IHC detects specific antigens (protein ±carbohydrate) using antigen-antibody recognition.
Epitope—exact part of the antigen molecule with which the antibody combines.
In practice, mostly done on formalin-fixed paraffin-embedded (FFPE) tissue.

Pre-Staining Steps:
1) Antigen Retrieval first to "unmask" certain antigens altered by fixation.
A common method of antigen retrieval is heating (e.g., in a microwave).

2) Blocking Nonspecific Background Staining: The tissue is treated with serum or other antibodies to reduce nonspecific binding and agents to reduce endogenous enzyme activity.

Detection Systems:
To make antibodies visible by light microscopy, they must be labeled or flagged.
These could be florescent, but more commonly are conjugated enzymes that create a visible chromogen signal through a precipitating chemical reaction, like peroxidase.



***Direct*-Conjugate-Labeled Antibody Method**
The signal is applied to the antibody that directly detects the epitope (primary antibody).
Pro: Quick (one reaction)
Con: Requires more (expensive) primary antibody and conjugations

***Indirect* Procedure (more common now)**
The signal is applied to a secondary antibody that detects the primary antibody.
Pro: More versatile (only secondary antibody has to be conjugated), needs less expensive primary antibody.
Con: Takes longer (more steps)

Avidin-biotin complexing and polymer-based amplification can also be used to attach a signal to the primary or secondary antibody.

Basic Info

There is an existing, awesome, brief, but also fairly comprehensive, resource about IHC: "Quick Reference Handbook for Surgical Pathologists" by Rekhtman et al. So, please check this out for more exhaustive details and lists. Also, there is a lot of IHC info sprinkled throughout the rest of my notes with additional important contextual information.

Herein, I've tried to stick to basic, important info not covered elsewhere in my notes, while also not just copying all of this other fantastic resource.

Monoclonal vs Polyclonal

Monoclonal antibodies —As a single clone, they all recognize the same single epitope (more specific). Made using "hybridoma."

Polyclonal antibodies— As multiple clones, they recognize multiple epitopes on the same antigen (more sensitive, less specific). Made using antisera immunized into an animal. Higher background staining.

Most current commercial antibodies are mouse or rabbit monoclonal antibodies.

Localization

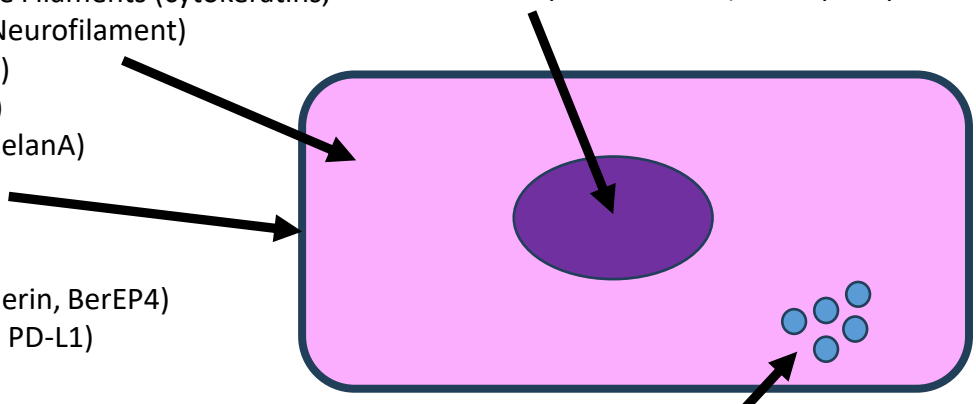
Always remember where a stain should localize to: Location, Location, Location!

Cytoplasmic
Cytoskeleton: Intermediate Filaments (cytokeratins, Vimentin, Desmin, GFAP, Neurofilament)
Contractile proteins (Actin)
Secretory products (ACTH)
Melanosomes (HMB45, MelanA)

Membranous
CDs: CD20, CD3, etc…
Adhesion proteins (E-cadherin, BerEP4)
Signaling receptors (HER2, PD-L1)

Nuclear AND Cytoplasmic
S100, p16, Calretinin,

Nuclear
Transcription Factors (TTF-1, p53, p40, etc..)



Granular Cytoplasmic
Localization to cytoplasmic organelles
NapsinA, AMACR, Hepar1

(Perinuclear) Cytoplasmic "Dot-like"
Cytokeratin in neuroendocrine neoplasms
CD30 and CD15 in Hodgkin lymphoma

***Unusual* localization can be useful too:**
TTF1 cytoplasmic localization is seen in tissue with hepatic differentiation (including normal liver).

Membranous **Ki67** can be seen with Hyalinizing Trabecular Tumor.

Cytoplasmic **WT-1** can be seen with vascular tumors.

Modified from: "Quick Reference Handbook for Surgical Pathologists" by Rekhtman et al. 2019.

Basic **Differentiation** Markers

Modified from: "Quick Reference Handbook for Surgical Pathologists" by Rekhtman et al. 2019.

Differentiation	Stains
Epithelial	Cytokeratin, EMA, BerEP4, Moc31, Claudin-4
Mesothelial	D2-40, Calretinin, WT-1, Cytokeratin
Myoepithelial	Cytokeratin, SMA, Calponin, S100, SOX10, p63, p40, GFAP
Smooth Muscle	Desmin, SMA, muscle-specific actin, SMM-HC, Calponin, H-Caldesmon
Skeletal Muscle	Myogenin, MyoD1, Muscle-specific actin, Desmin
Myofibroblastic	Actins (SMA, MSA) in a "tram-track" appearance (partial smooth muscle)
Neuroendocrine	Synaptophysin, Chromogranin, CD56, INSM1, Cytokeratin (perinuclear dot-like)
Germ Cell	SALL4, PLAP
Melanocytic	S100, SOX10, HMB45, Melan-A (MART1), MITF, tyrosinase
Endothelial	CD31, CD34, ERG, Fli-1, D2-40 (lymphatic)
Schwann cell	S100, SOX10
Glial	GFAP, OLIG2
Neuronal	Neurofilament, NeuN, Synaptophysin
Hematopoietic	Pan-hematopoietic: CD45 (LCA) B cell: CD20, PAX5, CD19, CD79a T cell: CD3, CD43 Plasma cell: CD138 Myeloid: CD43, CD117/c-kit, CD34, MPO
Histiocytes	CD68, CD163
Mesenchymal	Vimentin *(Historical: Not actually really used clinically often)*
Adipocytes	S100 *(Often not necessary)*

Cytokeratins *"Keratins" or "CK"*

Cytoskeletal intermediate filaments that are often considered the most fundamental marker of **epithelial differentiation**. Notably though, they can get many epitheli*oid* cells (e.g., epithelioid sarcoma)

Numbered 1-20, but for practical purposes can be thought of as "High" and "Low" molecular weight.

High Molecular Weight Cytokeratin (HMWCK): Squamous (and spindled) epithelial cells
Expressed more in squamous and spindled epithelial cells and myoepithelial cells. More structural.

Low Molecular Weight Cytokeratin (LMWCK): Simple (non-squamous) epithelial cells.
Expressed more in visceral organs and glands. Less structural.

To increase sensitivity, keratin stains are often combined in "***Cocktails***," such as:
AE1/AE3, OSCAR, PANK (MNF-116): Very broad, good screening CKs, include most keratins
CAM5.2: LMWCKs (CK7 and CK8)
CK903 (34βE12): HMWCKs (CK 1, 5, 10, 14)
CK5/6: HMWCK

Muscle markers

Desmin: A good "pan-muscle" marker (+ in all types of muscle). Good for screening.
Warning: Some none muscle tumors/tissue can stain with Desmin (e.g., desmoplastic small round cell tumor, mesothelium, Wilms).

Myogenin & MyoD1: Very specific to skeletal muscle. Nuclear transcription factors.

Calponin, h-Caldesmon, and smooth muscle myosin heavy chain (SMMHC): Smooth muscle markers.
It's sometimes necessary to get a few (or all) in poorly differentiated leiomyosarcomas.

Myoepithelial cells: Show both epithelial and smooth muscle differentiation. Therefore, they express both epithelial (CK) and muscle (e.g., SMA, Calponin) markers. They also often express S100, GFAP, and/or p63/p40.

Myofibroblastic cells: Show both fibroblastic and *incomplete* smooth muscle differentiation. Show partial/weak expression of smooth muscle markers, often in a peripheral "tram track" pattern.

	Desmin	MSA (HHF-35)	SMA	Calponin	h-Caldesmon	SMMHC	Myogenin & MyoD1
Skeletal Muscle	+	+	-	-	-	-	+
Smooth Muscle and Myoepithelial cells	+	+	+	+	+	+	-
Myofibroblast	+/-	+/-	+	+/-	-	-	-

Modified from: "Quick Reference Handbook for Surgical Pathologists" by Rekhtman et al. 2019.

Melanocyte markers

S100 & SOX10: Most sensitive/broad markers of melanocytes (including desmoplastic melanoma, which are often negative for markers below). Also stain neural and myoepithelial tumors.

Melan-A (MART-1): Cytoplasmic. Also gets adrenal and sex cord stromal tumors.
HMB45: Stains pre-melanosomes cytoplasmically. Can help differentiate nevus vs melanoma (see Melanocytic tumor notes). Positive in PEComa's.
MITF: Nuclear stain. Less sensitive and specific (get's lots of other things in the dermis).

Sensitivity: SOX10, S100 >> Melan-A > HMB45; **Specificity:** HMB45 > Melan-A > SOX10 > S100

Neuroendocrine (NE) markers

Synaptophysin (*"Synapto"*) **& Chromogranin** (*"Chromo"*): Main markers of NE differentiation.
Stain neurosecretory granules→ cytoplasmic granular staining.
Generally, Synaptophysin is more sensitive. Chromogranin is more specific.

INSM1: Newer NE maker (so data still accumulating). Tentatively thought to be more sensitive and specific. Nuclear transcription factor.

CD56: Most sensitive NE marker, but least specific (also gets NK-cells, etc.)
NSE (Neuron-Specific Enolase): Not used much anymore due to low specificity (Non-specific enolase ;-)

In practice, most well-differentiated NE things stain with Synaptophysin and Chromogranin.
INSM1 and CD56 are most useful in poorly-differentiated NE carcinomas (that may lose synapto/chromo)

Vascular markers

All blood vessel and lymphatic endothelium stain with CD31 and ERG.
Lymphatics stain with D2-40, but often not CD34.

ERG: Nuclear. Pretty specific. Also gets *some* prostate cancer, epithelioid sarcoma, Ewing sarcoma, hematolymphoid.

CD31: Cytoplasmic and membranous. Also gets megakaryocytes and macrophages.

CD34: Cytoplasmic and membranous. Least specific. Also gets DFSP, many fibroblasts/stromal cells, SFT, GIST, Nerve sheath tumors, Epithelioid sarcoma, Myeloid neoplasms, Blasts, etc…

D2-40: Membranous. Lymphatic endothelial cells. Also gets mesothelium, seminoma/germinoma, follicular dendritic cell sarcoma, etc..

Is ***that*** positive?

***Pro-tip*: Check the positive and negative external controls (and any internal controls) and use these as your guide.**

Sometimes, staining is obviously positive or negative, but sometimes it's not.

You do NOT need to report as binary positive/negative, particularly if it's unclear.

I often use adjectives (e.g., focal, weak, patchy, rare, strong, etc…) with my thoughts (e.g., "Interpreted as ____"), if appropriate. (See examples→)

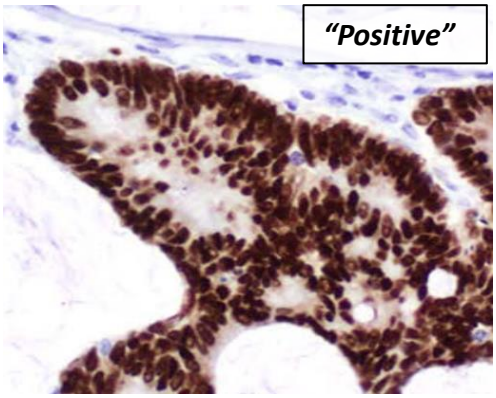
While perhaps this is cheating a little, many pathologists will err in the side of their favored diagnosis that fits better clinically when it comes to interpretation. For example, if they expect a stain to be negative, but there is rare staining, they may say "interpreted as negative [or equivocal]" or "Not clearly positive."

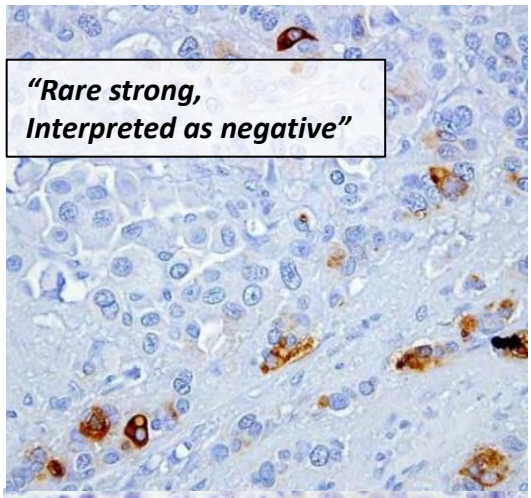
What if two stains "disagree" (support conflicting conclusions)?!

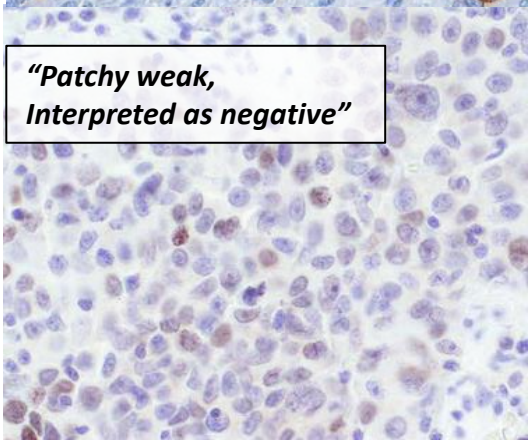
1) Consider doing another round as a "tie breaker" by getting more data.

2) The stronger stain often "wins"

3) In truly uncertain cases, molecular testing could yield helpful information.







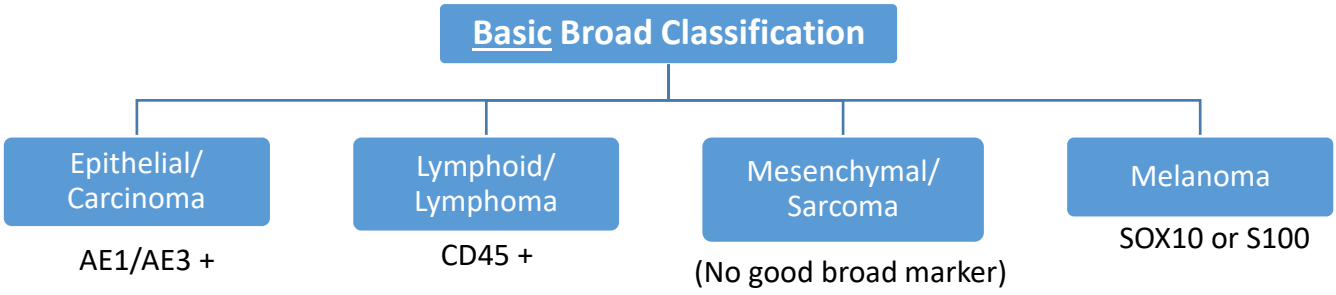
Commonly Used Stains to Know: *(excluding many of those just discussed)*

Stain	Positive in	Stain pattern/type
p40 (and p63)	Squamous, Urothelial, Basal, and Myoepithelial cells. p40 is more specific.	Nuclear transcription factor
CK5/6	Squamous, Urothelial, Mesothelial, and Myoepithelial cells.	Cytoplasmic
PAX8	Müllerian (GYN), Thyroid, Kidney. With polyclonal antibody: Thymus, pancreatic NET	Nuclear transcription factor
GATA-3	Breast and Urothelial. Also: Paraganglion, Choriocarcinoma, Mesonephric, Parathyroid. Some kidney, etc…	Nuclear transcription factor
Mammaglobin	Breast (Medium specificity, low sensitivity), salivary, sweat glands	Cytoplasmic
GCDFP-15 (BRST2)	Breast (high specificity, low sensitivity), salivary, skin adnexal tumors	Cytoplasmic
WT-1 (N-terminus)	Mesothelium, Serous GYN tumors. Wilms. CIC-DUX4.	Nuclear (cytoplasmic can be seen in many tumors)
TTF-1	Lung adenocarcinoma, Thyroid. Small cell carcinoma from any site.	Nuclear Transcription Factor
Napsin-A	Lung (adenoCA), RCC (especially papillary), GYN clear-cell CA	Granular cytoplasmic
Thyroglobulin	Thyroid	Cytoplasm (and colloid)
CDX2	Mucinous and/or enteric adenocarcinomas. Heterogeneous in Pancreatobiliary. Small bowel NET.	Nuclear Transcription Factor
SATB2	Colorectal origin of adenocarcinoma, osteoblastic lineage. Rectal NET, BCOR-rearranged sarcoma	Nuclear Transcription Factor
NKX3.1	Prostate (most sensitive & specific). Some salivary gland	Nuclear Transcription Factor
PSA, PSMA, and PSAP	Prostate (less sensitive/specific). Some salivary gland.	Cytoplasm and/or Membranous
ERG	Vascular neoplasms; also expressed by subset of prostate cancer, Ewing sarcoma, Epithelioid sarcoma and Acute leukemia;	Nuclear Transcription Factor
SALL4	Germ cell neoplasia; Aberrant expression in a significant minority (20-30%) of serous, gastric, urothelial, hepatoid, and biliary carcinomas	Nuclear Transcription Factor
SF1	Adrenal cortical and sex cord-stromal tumors.	Nuclear Transcription Factor
Inhibin	Arenal cortical and sex cord-stromal tumors.	Granular cytoplasmic
Calretinin	Mesothelium. Adrenal. Sex-cord stromal. Mesonephric. Ganglion.	Nuclear and cytoplasmic
SOX10	Melanocytic, nerve sheath, and myoepithelial tumors; also often (60%) expressed by triple-negative breast cancer	Nuclear Transcription Factor
S100	Melanocytic, nerve sheath, and myoepithelial tumors; Langerhans cells.	Nuclear and cytoplasmic
Hepar1 & Arginase	Hepatocellular differentiation	Cytoplasmic
ER	Breast, GYN (endometrioid ≫ serous). Can label subset of other CAs (e.g., ~5% of lung CAs are ER+)	Nuclear Transcription Factor

Unknown Tumor/Metastasis Work-up

"The Gut Course"

Always think broadly and first try to put things into a "bucket," then you can get more specific after. Most tumors encountered in surgical pathology fall into one of these general buckets.



Metastatic Carcinoma of Unknown Origin

The diagnosis of carcinoma is supported by **cohesive growth** and **expression of epithelial marker**(s).
Epithelial markers: Cytokeratin, EMA, BerEP4, Moc31, Claudin-4

Always consider the clinical setting: Age, Gender, Location

Look for evidence of *squamous* or *glandular differentiation*, which can narrow your DDX and suggest different next steps in work up.

Especially if it's in a lymph node (LN), be sure to consider and rule out lymphoma.

Demographic	Most Common Primaries
Men	Prostate, Lung, Colon
Women	Breast, Lung, Colon, GYN
Kids	(First, always consider heme!) Neuroblastoma, Rhabdomyosarcoma, Kidney
Teen/Young adult	Germ cell tumors, Thyroid, Breast, Colon
"Occult" primary	Pancreas, Lung, Stomach

Site of Metastasis	Most Common Primaries
Bone	Breast, Lung, Thyroid, Kidney, Prostate ("*BLT with a Kosher Pickle*")
Liver	Colon, upper GI (including Pancreatobiliary), Breast, Lung, Melanoma
Lung	GI (including colon and upper), Breast, Kidney, Melanoma
Peritoneum	GYN, GI
Pleura	Lung, Breast
Neck LN	SCC (HPV±), Thyroid (PTC)
Intra-parotid LN	Scalp SCC, Melanoma, Merkel. Salivary.
Axillary LN	Breast, Lung, Melanoma
Inguinal LN	Lower extremity (SCC, Melanoma), GYN (Vulva, Cervix), Anorectal, GU
Paraumbilical or Left supraclavicular LN	Visceral organs (e.g., Stomach) (Sister Mary Joseph and Virchow's nodes, respectively)

Making an IHC Panel

There are certain situations where I almost always use the same preselected IHC pattern. For example, looking for metastatic carcinoma in pleural fluid (BerEP4, D2-40, CD68, to start with), or carcinoma in the lung to determine if it's SCC or adenocarcinoma (p40 and TTF1), where you have the same DDX. However, in most other circumstances (e.g., working up a liver metastasis), I use a panel customized to the situation as my DDX depends on the patient history and demographics.

Here is a general approach to developing a panel:

1) Consider the clinical scenario
Before even looking at the slide, what is your DDX based on the patient demographics and presentation? Do then have a known history of malignancy? Come up with a list of your top few considerations.

2) Incorporate morphology
Now, look at the slide. Does it match with your DDX? Anything to add or remove? Does it look "classic" for anything? Consider pulling the slides for any prior tumors for morphologic comparison (and if they match maybe not doing any/many stains).

3) Pick a *limited panel* to start that addresses your top DDX.
It's often best to have some stain(s) you expect to be positive and expect to be negative.
There are relatively few circumstances where you should just do a single stain for diagnostic reasons. If you only do one stain, you're more likely to be led astray by aberrant staining.

I often start with **1 or 2 stains for each diagnosis**. I also tend to personally favor nuclear transcription factors as they are often easier to interpret. I often have several unstained slides cut simultaneously for any follow up panels to save tissue. If it's an urgent scenario (e.g., a rapidly growing mediastinal mass in a kid and it's almost the weekend) feel free to order a big panel though!

4) Do follow up panels as necessary to get to a specific diagnosis.
The first panel can often go two ways:

A) It gets you (at least part way) to a specific diagnosis, in which case you may be done, or order follow up stains to get even more specific, further support your diagnosis (if it's unexpected), or for prognostic purposes.

B) It totally fails: Sometimes everything is negative, or you get a mixed picture with points for and against a diagnosis.

i) If everything is negative, then start back at the beginning with an even broader panel with screening antibodies (e.g., AE1/AE3, S100, CD45) and consider mimickers like melanoma.

ii) If you get a mixed picture, add stains that specifically target the new dilemma.

Example: 50-year-old women with multiple omental masses and no known history of malignancy.

My thought process: A)Multiple masses are usually metastasis. The most common mets to the omentum are 1) GYN and 2) GI (upper and lower). Less likely is lung or breast.

B) Morphology looks like adenocarcinoma, likely not colon (no "dirty necrosis"), so there is no question as to if it's carcinoma, therefore a broad-spectrum cytokeratin is likely unnecessary (unless things don't work later on).

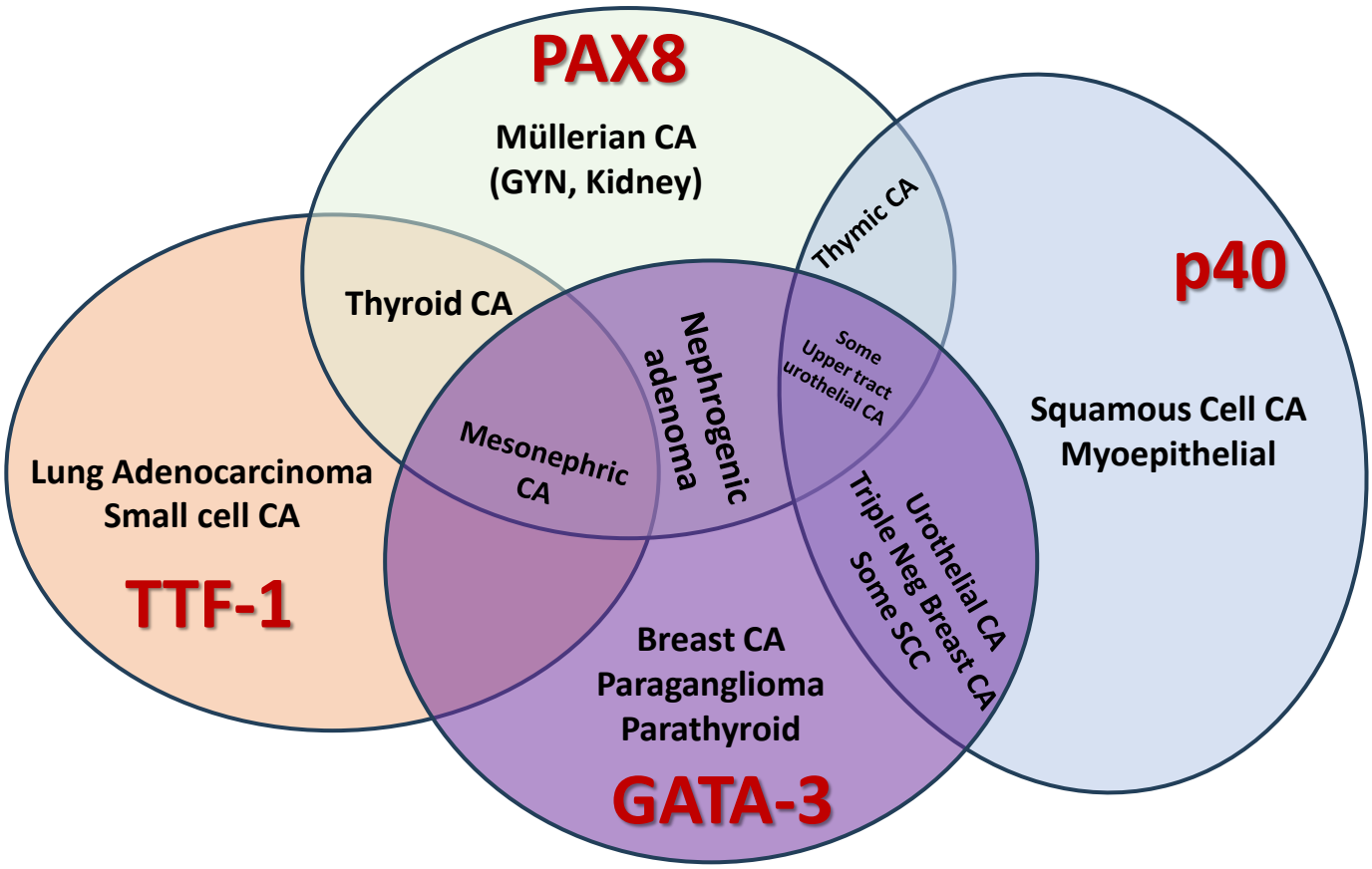
C) My initial panel: CK7, CK20, CDX2, SATB2, PAX8, ~5 unstained slides for a potential second panel
This panel includes a stain or two that would be positive and/or negative in each of the main diagnoses I'm considering, Mets from the pancreas, stomach, colon, ovary, and uterus.

Venn Diagram's of IHC staining

I hope to illustrate with my figure below, that there is **a lot of overlap** between markers of origin/differentiation. There is also a lot of "aberrant" (nonspecific, unexpected) staining (see below).

So, **I almost always do a at least 2 stains while determining differentiation/origin**—at least one for every DX I'm considering, with some expected positive and negative ones. Doing a more than just one stain helps keep pitfalls in check. However, it's a balance, too many stains is expensive and can cause confusion. So, don't go too crazy! ;-)

With your IHC panel, try to create a Venn diagram that supports your diagnosis (and lessens the likelihood of others).



Musings on specificity: The parable of GATA-3

As you start consuming the pathology literature, you'll notice this recurrent pattern: A new IHC stain is developed and touted as ground breakingly specific. Then, after it finds its way into practice, it quickly becomes clear that it *actually* stains a lot of things. For example, the "multi-specific" breast and urothelial marker, GATA-3, actually stains about half of all pancreatic adenocarcinomas, some lung cancers, and a plethora of other things.

This has led to the humorous aphorism, ***"When a new stain comes out, you have to use it quick while it's still specific!"*** ;-)

CK7 and CK20

The LMWCKs CK7 and CK20 can be used in conjunction to potentially support a site of origin given their different expression in different tumors/organs.

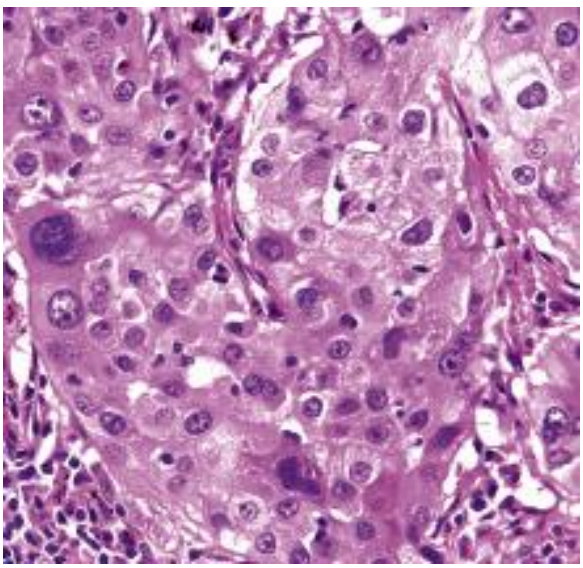
In my practice though, I've found these of limited utility as you can see many common tumors are CK7+/CK20-. So, while there are definitely circumstances when I'll employ them, I more commonly rely on more specific markers, particularly nuclear transcription factors (e.g., TTF1, p40, GATA3, PAX8), which are often "cleaner" and easier to interpret.

Typical CK7 & CK20 expression

Note: There are many exceptions, refer to more exhaustive resources for more info.
Like this one (PMID: 35390310).

	CK7+	CK7-
CK20 +	**CK7+/CK20+ (Double ++)** Peri-diaphragmatic GI organs (pancreas, biliary tree, stomach) and bladder	**CK7-/CK20+** Colon Merkel Cell
CK20 -	**CK7+/CK20-** Above-the-diaphragm organs (lung, breast, thyroid, salivary gland) and female GYN tract (uterus, ovary)	**CK7-/CK20- (Double --)** Liver, Kidney, Prostate, Germ cell tumor, Adrenal, Squamous, most Neuroendocrine

Metastatic Undifferentiated Carcinoma



Epithelial tumors (cohesive with expression of epithelial markers) without clear glandular or squamous growth.

Often has nonspecific, large, polygonal morphology.

First, "***When you hear hoofbeats, think horses, not zebras.***" Using the previously mentioned methods, consider the common "horse" diagnoses, like lung cancer, breast cancer, bladder cancer, GI cancer, SCC, thyroid, etc…

Possible IHC panel to use:
TTF1, GATA3, p40, PAX8, CDX2, ±CK7&CK20

"Common zebras" to consider:
Hepatocellular carcinoma (HCC) → Consider Hepar1, Arginase, Glypican-3
Adrenal Cortical Carcinoma → Consider SF1, Melan-A, Inhibin, Calretinin
Triple Negative Breast Cancer (TNBC) → Consider SOX10
Large cell neuroendocrine carcinoma and NET variants → Consider INSM1, Synaptophysin, Chromogranin
Melanoma (rarely expresses some epithelial markers) → Consider S100, SOX10

Adenocarcinoma in the Liver

Adenocarcinoma = Gland formation

In the liver, **metastases are more common than primary tumors.** (particularly in non-cirrhotic livers)

Factors that strongly **favor a metastasis**: **Multiple tumors**, History of prior malignancy

Most common sites of origin: Colorectum, pancreas, stomach, breast, lung, kidney, melanoma. Despite prostate cancer being very common, it rarely goes to the liver.

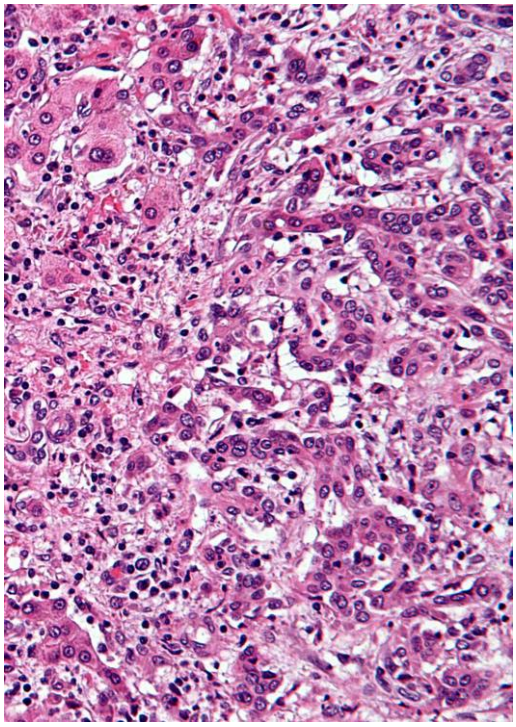
The age-old conundrum: Pancreatic Carcinoma Metastasis vs Primary Intrahepatic Cholangiocarcinoma'

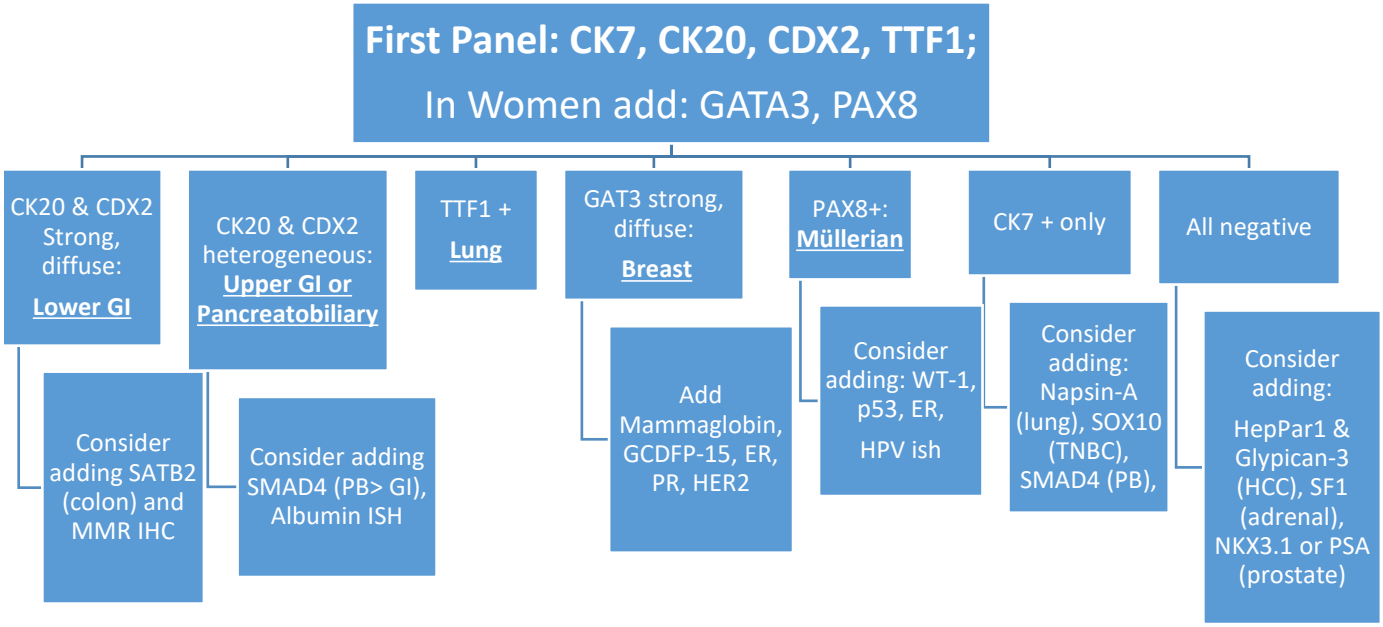
Both of these tumors are "*Pancreatobiliary*" (PB) and derive from the essentially the same cell type: ductal epithelium from the bile ducts and intrahepatic bile ducts.

Accordingly, they have identical/overlapping IHC profiles:
CK7+, CK20±, CDX2±

Although not entirely specific, positive Albumin ISH appears to strongly favor a primary intrahepatic cholangiocarcinoma, as does loss of BAP1 by IHC.

That said, given what seems to be imperfect specificity, the likely best discriminator is good clinical and radiographic correlation. ;-) If there is a pancreatic mass→ it's likely a met!





Modified from: Bellizzi AM. Adv Anat Pathol. 2020 May;27(3):114-163. PMID: 32205473

Molecular alteration-specific IHC stains

Tumors are getting increasingly defined by specific genetic alterations (amplifications, deletions, mutations) and gene fusions.

Simultaneously, new antibodies are being developed to recognize many of these alterations. Some refer to this as "***Next-generation immunohistochemistry***." IHC has the advantage of being much faster and cheaper than most genetic analyses, so there will likely be an increasing trend to utilize these in practice.

Examples are becoming increasingly numerous and include:
p53, RB1, SWI/SNF (INI1, BIRG1), SSX-SS18, STAT6, DDIT3, FOSB, CAMTA1, BAP1, H3K36M, H3G34W, H3K27me3, ALK, BRAF V600E, PDGFRA, BCOR, TRK, YAP1, SDH-B, MYC, etc…

What if a tumor is ***negative*** for ***everything***?!!

Sometimes, tumors are just so anaplastic, that all we can say is ***"undifferentiated malignant neoplasm."***

However, before "throwing in the towel," consider the following:

1) Loss of antigenicity due to poor fixation/processing (particularly if there is no good internal control). One way to investigate this is with vimentin IHC, which is so non-specific that it stains just about everything (especially if it is vaguely spindled/mesenchymal). If Vimentin is negative, antigenicity may be the issue and you may need better fixed tissue for analysis. As Dr. Richard Kempson would say, "*It stains with Vimentin; well, we know it's mammalian*" (although I bet avian tissue may stain too! ;-)

2) Rarer lines of differentiation. Consider the diagnoses below and stains like: p40, ERG, CD34, Desmin, CD30, CD99, Myogenin, Synaptophysin, SF1, more heme markers, etc…

Diagnosis	Stains to consider for Dx
Sarcomatoid carcinoma	HMWCKs, p40
Poorly-differentiated neuroendocrine carcinoma	Synaptophysin, Chromogranin, INSM1, TTF1, Rb
Adrenal cortical carcinoma	SF1, Melan-A, Calretinin, Inhibin, Synaptophysin
Sarcoma	CD34 (rarely expressed by carcinomas), MDM2, SMA, Desmin,
GIST	CD117, DOG1, CD34
Follicular dendritic cell sarcoma	CD21, CD23, CD35
Acute leukemia/lymphoma	CD34, TdT, CD43
Large cell lymphoma	ALK, CD30
Plasma cell neoplasms	CD138, CD79a, MUM1, kappa/lambda
Hodgkin lymphoma	CD30, CD15, PAX5
Plasmablastic lymphoma	CD79a, CD138, MUM1, EBVish
Melanoma	SOX10, BRAF V600E
Germ cell tumor	SALL4, PLAP
Pheochromocytoma/Paraganglioma	Synaptophysin, Chromogranin, GATA3, INSM1

Modified from a presentation by Dr. Andrew Bellizzi, University of Iowa, USCAP, 2021.

Laboratory-Developed vs. Ready-to-Use Approaches

There are two main types of IHC protocols:

	Laboratory-Developed Tests (LDTs) "Home-brewed" protocols	Commercially developed "Ready-to-Use" Protocols
General	All antibodies, reagents, dilutions, chromogens, etc… individually, locally optimized	Purchased kits with all reagents and protocols provided and pre-optimized
Pro's	Customization Cheaper Can be done on any staining platform	Easier to use (less to figure out) Often FDA-approved Requires less validation
Con's	Non-FDA approved Requires extensive validation Requires more expertise	Often platform-specific (e.g., Ventana, Leica, Dako, etc…) No customization More expensive More limited shelf-life

Commentary: Historically, many labs, particularly academic and larger labs, have used at least some LDTs. However, there is increasing regulation in this space in the United States with the FDA currently trying to transition everyone to FDA-approved (commercially developed) protocols. This is controversial, with many pathologists unhappy with this forced transition.

Quality Assurance

The goal of QA is to ensure **standard quality between laboratories**.

Daily Controls:

Positive Control—Run in parallel to validate that the appropriate antibody-antigen reaction has occurred. Nonpatient tissue or cells containing antigen to be detected and quantified (processed in the same way). Known expected result, ideally low and moderate intensities. Validates all steps of analysis, including training user for appearance and localization.

Negative Control—Patient tissue with components that are the same as tissue to be studied. Protocol leaves out antibody. Allows to evaluate background staining and tissues with endogenous pigments (e.g., melanin, hemosiderin, and lipofuscin).

Within a patient sample, there can also be areas of *internal* positive and negative control. That is, areas of the tissue that are expected to be inherently negative/positive (e.g., a nerve or melanocytes with S100). This can allow one to look validate the protocol in the patient tissue itself and also look for unexpected cross-reactivity.

Principles of New IHC Validation: (Based on CAP Guidelines)
Full validation is beyond the scope of these notes, but, generally:

- Validation sets should use similar fixatives and conditions as clinical samples (e.g., decal) .
- Labs should use at least 10 positive and 10 negative controls when validating a new stain (and 20 of each for predictive/prognostic markers, like HER2, ER, etc…)

Predictive/Prognostic Markers

Staining results can be ***predictive***, indicating whether a tumor is likely to respond to a particular therapy, or ***prognostic***, providing information about the likely course and outcome of a disease (or, sometimes, both!).

Examples of this are sprinkled through out my notes. For example, in the breast section there are guides to ER, PR, and HER2 interpretation. In the Esophagus tumor notes, there is a guide to GI HER2 interpretation (which is different than in the breast—just to keep you on your toes!)

Below, I've included a guide to some stains (so far just PD-L1) that are applicable to multiple sites.

Generally, given that their interpretation can play a big role in therapy, these tests are more tightly regulated by the FDA and CAP and require additional validation and also proficiency testing.

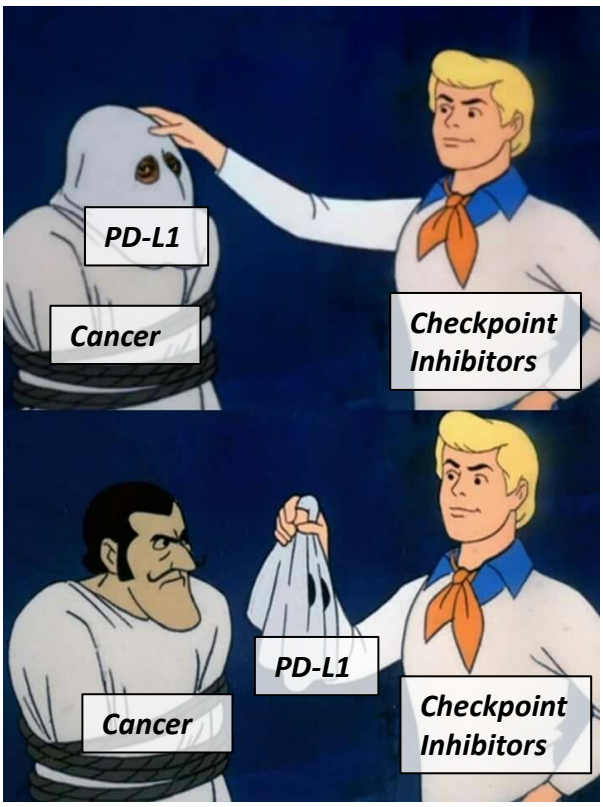
PD-L1 and Immunotherapy

Immunotherapy uses a patient's own immune system to fight cancer.

Several strategies:
Adoptive cellular (T cell) therapy: Utilizes T cells that target the tumor (either through engineering or selection of tumor infiltrating lymphocytes). Examples: CAR T cell and CAR NK T cell

Cancer vaccines, Cytokine therapy, Monoclonal antibodies (mark the cancer as a target for the immune system, or boost the ability of immune cells to fight the cancer)

Checkpoint inhibitors: block inhibitory receptors used by tumors to dampen anti-tumor T-cell response. Most widely used immunotherapy.



Under normal circumstances, "**immune checkpoints**" are inhibitory regulators of the immune system that are crucial for maintaining self-tolerance and preventing autoimmunity.

Examples: CTLA4 and programmed cell death 1 (PD-1)/ programmed cell death ligand 1 (PD-L1)

Some tumor cells express the inhibitory checkpoint proteins, PD-1, PD-L1, or CTLA-4, as a means of suppressing antitumor T-cell responses. *(I think of this as a mask the tumor cells on to disguise themselves as "normal" cells)*

Immune checkpoint inhibitors bind to these inhibitory proteins, blocking this inhibitory (tumor protecting) signal, allowing T-cells to stay active and attack the cancer cells. *(I think of this as a drug that takes off the tumors "self/normal" mask and exposes it for what it is.)*

Examples:
anti-PD-1 antibody: Pembrolizumab, Nivolumab, Cemiplimab
anti-PD-L1 antibodies: Atezolizumab, Avelumab, Durvalumab
CTLA 4 inhibitor: Ipilimumab

PD-L1 and Immunotherapy *(Continued…)*

Therapy	Corresponding PD-L1 IHC
Nivolumab	28-8 (Dako)
Pembrolizumab	22C3 (Dako)
Atezolizumab	SP142 (Ventana)
Durvalumab	SP263 (Ventana)
Avelumab	73-10 (Dako)

In some instances, these drugs have dramatic, durable treatment response and are essentially a "cure." However, in many cases, tumors show little/no response. Some people also experience significant immune-related side effects (like autoimmune GI disease, simulating IBD). And, notably, these drugs are incredibly expensive.

For these reasons, to better determine who would benefit from immunotherapy, **PD-L1 protein expression on tumor and/or immune cells has emerged as the predictive biomarker for sensitivity to immune checkpoint blockade therapy.**

Unfortunately, the clinical trials for these drugs all used different antibody clones, so the FDA approved the drugs with their own specific "*companion diagnostic*" prepackaged stain kits which use different IHC platforms, and different grading cutoffs (because…. umm… reasons… *[gestures broadly at corporate self interest]* ;-)

For example, for pembrolizumab, which seems to be the most popular of the drugs, the companion test is PD-L1 IHC 22C3 pharmDx, which is only available in a kit for Dako/Agilent stainers.

The Blueprint PD-L1 IHC Assay Comparison Project revealed that three of the four assays (22C3, 28-8, and SP263) were closely aligned on tumor cell staining whereas the fourth (SP142) showed consistently fewer tumor cells stained. All of the assays demonstrated immune cell staining, but with greater variability than with tumor cell staining. *(PMID: 27913228)*

PD-L1 IHC Interpretation *(clone **22C3** for Pembrolizumab)*

Need to evaluate **at least 100 viable Tumor Cells**

Tumor cells that stain show membranous staining of any intensity are considered positive. Does not have to be circumferential.

In **tumor-infiltrating immune cells,** membrane, as well as cytoplasmic staining, is considered positive. Histiocytes/macrophages may express PDL-1, and are not counted if they are just in a lumen/space.

Tumor Proportion Score (TPS): Used in Non-small cell lung cancer only.

% of viable tumor cells showing partial or complete membrane staining (≥ 1+) relative to all viable tumor cells present in the sample (positive and negative). Ignore cytoplasmic staining.

Three levels based on a Tumor Proportion Score (TPS):
– TPS < 1%: No PD-L1 expression → *(No benefit)*
– TPS 1–49%: PD-L1 expression → *(some possible benefit)*
– TPS ≥ 50%: High PD-L1 expression → *(Most benefit)*

$$\text{TPS} = \frac{\text{\# PD-L1 positive tumor cells}}{\text{Total \# of PD-L1 positive + PD-L1 negative tumor cells}}$$

Combined Positive Score (CPS): Used in *all* other cancers (everything but lung)

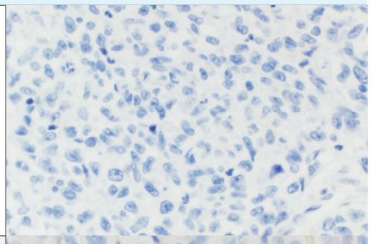
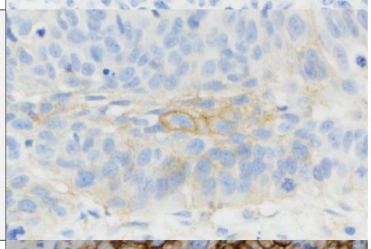
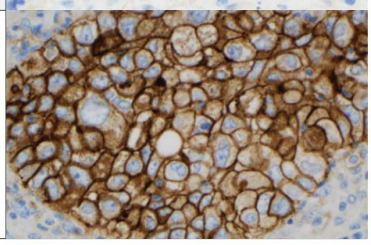
The number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. Although the result of the calculation can exceed 100, the maximum score is defined as CPS 100.

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells (tumor cells, lymphocytes, macrophages)}}{\text{Total \# viable tumor cells}} \times 100$$

Example of PD-L1 IHC TPS grading in NSCLC:

$$\text{TPS} = \frac{\text{\# PD-L1 positive tumor cells}}{\text{Total \# of PD-L1 positive + PD-L1 negative tumor cells}}$$

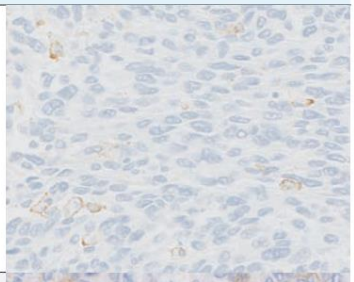
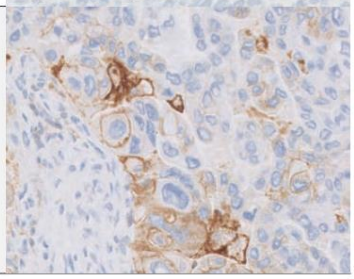
Table 1: TPS Expression Levels and Staining Characteristics

Expression Level	TPS	Staining Pattern	
No PD-L1 Expression	< 1%	Partial or complete cell membrane staining (≥ 1+) in < 1% of viable tumor cells	
PD-L1 Expression	1–49%	Partial or complete cell membrane staining (≥ 1+) in ≥ 1–49% of viable tumor cells	
High PD-L1 Expression	≥ 50%	Partial or complete cell membrane staining (≥ 1+) in ≥ 50% of viable tumor cells	

From: Dako/Agilent PD-L1 IHC 22C3 pharmDx Interpretation Manual – NSCLC

Example of PD-L1 IHC CPS grading in Urothelial Carcinoma:

Table 3: CPS and PD-L1 expression

CPS	PD-L1 Expression	Image (20×)
< 10	CPS is less than 10	
≥ 10	CPS is greater than or equal to 10	

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells (tumor cells, lymphocytes, macrophages)}}{\text{Total \# viable tumor cells}} \times 100$$

Tumor type	PD-L1 cutoff
NSCLC	TPS ≥1%
Gastric/GEJ Adenocarcinoma	CPS ≥1%
Esophageal SCC	CPS ≥10%
Cervical	CPS ≥1%
Urothelial	CPS ≥10%
Head and Neck SCC	CPS ≥1%
Triple Neg Breast	CPS ≥10%

From: Dako/Agilent PD-L1 IHC 22C3 pharmDx Interpretation Manual – Urothelial Carcinoma

Additional Resources

Websites:

The Protein Atlas: A *free* program that shows the expression of all of the human proteins in normal tissues and many tumors. Includes scanned images and statistics so you can see what stains with each antibody.
https://www.proteinatlas.org/

Immunoquery: A subscription-based service that lets you search for expression of different markers by tumor (and vice versa).
https://app.immunoquery.com/

Books:

"Quick Reference Handbook for Surgical Pathologists" Rekhtman, Baine, and Bishop. Springer. 2019.

"Diagnostic Immunohistochemistry: Theranostic and Genomic Applications. 6th Edition." Dabbs (ed.). Elsevier, 2021.

Articles:

Bellizzi AM. An Algorithmic Immunohistochemical Approach to Define Tumor Type and Assign Site of Origin. Adv Anat Pathol. 2020 May;27(3):114-163.