SEPTEMBER 2020 # 68

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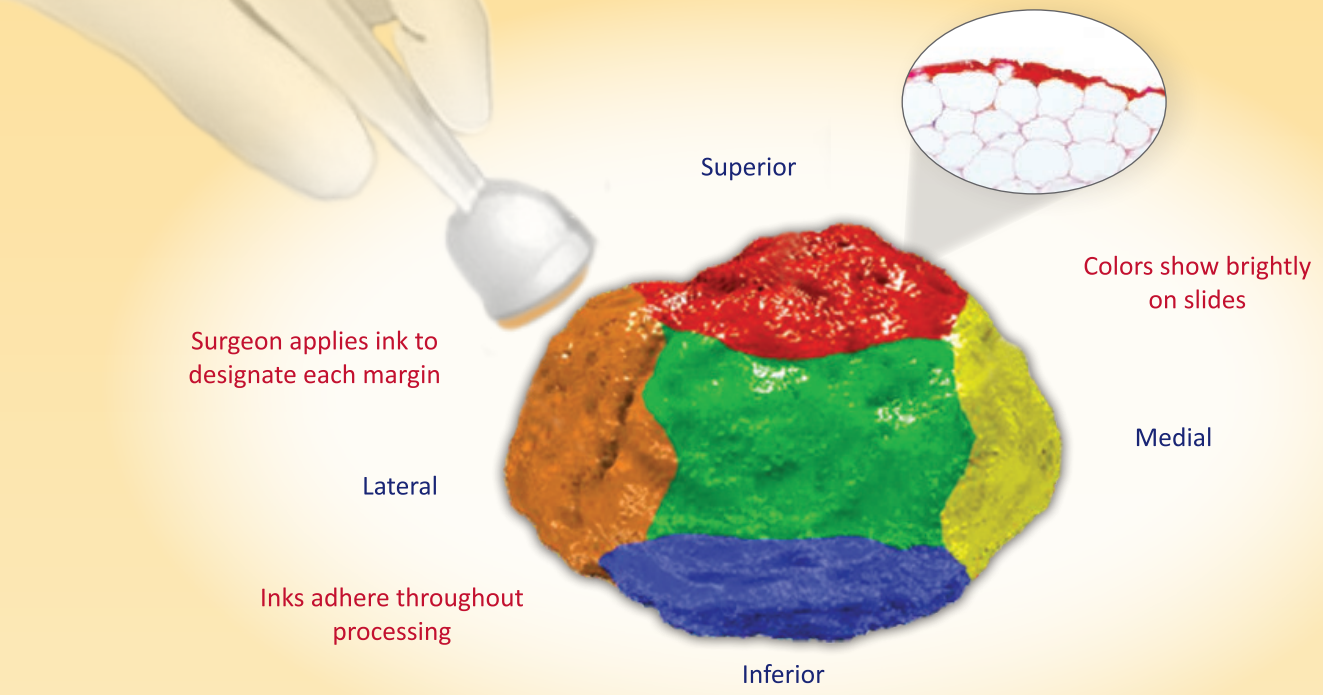
How pathology and molecular diagnostics serve our smallest patient population

MarginMarker™

Sterile Ink Kit for Intraoperative Use



Pathology Receives Specimens With Margins Clearly Marked by the Surgeon

In cancer surgery, the single most important predictor of local recurrence is the tissue margins.[1] Research shows discordance rates as high as 52% in the identification of specimen margins.[2,3] Re-excision rates exceed 20% in breast surgery.[4] Use of Vector Surgical's MarginMarker can result in:

- 50% fewer unnecessary re-excisions[1,5,6]
- Fewer delays of critical adjuvant therapy[7]
- More accurate re-excisions[5,8]
- Lower cancer recurrence[9,10]
- Improved cosmesis[6,8]

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VECTOR™ SURGICAL

Our grateful thanks to those who selflessly provide health care and support during these challenging times. Your service and dedication are sincerely appreciated.

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The Wisdom of Heraclitus

Our world is changing faster than ever – and we must not only keep up with it, but help others do the same

"The only constant in life is change," said Heraclitus. And although he made that declaration 2,500 years ago, it's no less true today, when uncertainty reigns supreme.

To open back up or lock back down? Pursue a COVID-19 vaccine or turn our focus to treatment over prevention? Spend as much time as possible in the lab or optimize our ability to work from home? These are the tough decisions we face now, often with no way of feeling sure of our choices. As doctors and scientists, we have the good fortune of both access to primary research and the background knowledge to understand it – but what about those who don't? With information (and misinformation) filling the airwaves, it's no surprise that many people aren't able to keep up with rapid developments in pandemic science – or the recommendations that follow.

So what can we do to help? As we reassess the risks and recommendations, we can ask ourselves, "How will I share this with people from different backgrounds?" We can challenge ourselves to communicate what we know – to educate, rather than alienate. We can seek out misunderstandings and offer clarity. And, sometimes, we can listen – and learn ourselves.

SPECIAL SERIES
Molecular Pathology

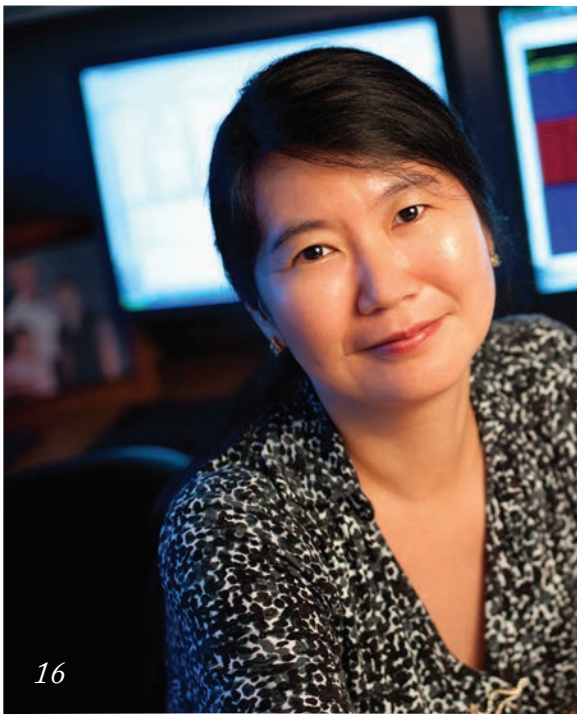
This issue of The Pathologist kicks off our special series on molecular pathology – another area in which we're seeing rapid change. New biomarkers, innovative diagnostics, and creative approaches are altering our understanding of disease on a daily basis. It's true that we aren't currently traveling to conferences to present these advances – but between virtual events, online publishing, and social media, we still have countless opportunities to share knowledge.

Has your communication style changed since the start of the pandemic? What are your top tips and tricks for your colleagues? Let us know (edit@thepathologist.com) and we'll share them with the world!

Michael Schubert
Editor

Contents

39



16

On The Cover

Two children administer medical treatments to their patient, Rufus the teddy bear.

Feature

In Practice

NextGen

Reports

Profession

Sitting Down With

the Pathologist

ISSUE 68 - SEPTEMBER 2020

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Upfront

Research
Innovation
Trends

To Catch a Cancer

Machine learning can help optimize liquid biopsy for rare circulating tumor cells

What if you could offer patients with cancer all the benefits of a biopsy – without the biopsy? That compelling idea – precision medicine with no need for invasive investigations – is what makes liquid biopsy such an attractive prospect. Unfortunately, there's a catch; circulating tumor cells (CTCs) are rare, presenting a detection challenge whose only solutions can be difficult and time-consuming. To expand the available options, one research collaboration has taken a new direction: computer assistance.

Unlike existing methods, the new approach requires no labeling or complicated microscopic techniques. Instead, a machine learning algorithm examines standard, low-resolution brightfield microscopy images to distinguish between CTCs and other cells (1). The scientists creating it ran two separate experiments: one on cultured cancer cell lines with a training set of 1,745 single-cell images; another on patient CTCs with a training set of just 95 images. The result? The algorithm exhibited an overall accuracy of 97.5 percent in the first experiment and 88 percent in the second.

"This study, though small, demonstrates that our method can achieve high accuracy on the identification of rare CTCs without the need for advanced devices or expert users," said senior author Yaling Liu in a recent press release (2). "With more data becoming available in the future, the machine learning model can be further improved and serve as an accurate and easy-to-use tool for CTC analysis."

So what's next for the researchers? They're not only training their algorithm with additional data, but also refining it to examine mutations in the DNA of CTCs. In addition, they're working on a microfluidic device to better capture and release CTCs (3) – all with the goal of fast, accurate, and minimally invasive personalized medicine for patients with challenging cancers.

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1. *S Wang et al., Sci Rep, 10, 12226 (2020). PMID: 32699281.*
2. *Lehigh University (2020). Available at: https://bit.ly/3g5eNV5.*
3. *Lehigh University (2020). Available at: https://bit.ly/3azhc9j.*

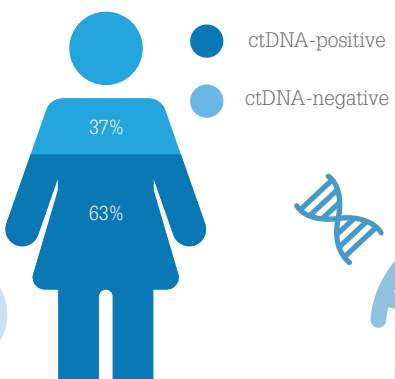
INFOGRAPHIC

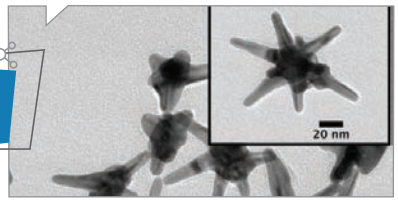
Circulating Secrets

What can circulating tumor DNA and cells tell us about disease recurrence and survival?

SPECIAL SERIES
Molecular Pathology

Examining the association between circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and distant disease-free survival (DDFS) in patients with early-stage triple-negative breast cancer.





SPECIAL SERIES
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QUICK HITS

The latest news and breakthroughs throughout diagnostic medicine

Gold star

Over 2,000 microRNAs have been linked to diseases ranging from brain tumors to Alzheimer's. A new method uses silver-plated gold nanostars to simultaneously identify multiple microRNAs from tissue samples without the need for labeling or target amplification. The technique could pave the way for more rapid detection of these early cancer biomarkers (1).

Effective antibodies?

To treat COVID-19, multiple trials are exploring the use of antibodies that can neutralize the virus. But how do we know they work? A team from New York's Rockefeller University have developed safer surrogate viruses that allow researchers to track infectivity and measure the success of potential neutralizing antibodies (2).

Sweat for success

Could sweat serve as a biomarker source? New research reveals that extracellular vesicles in sweat contain microRNA molecules – and that exercise changes their levels (3). Although sweat's unique microRNA content means it cannot be treated as a substitute for serum, the discovery may one day lead to noninvasive exercise monitoring.

Cardiac curveball

Autopsies of 22 confirmed deaths from COVID-19 have not revealed the expected signs of myocarditis-induced damage. Rather, patients exhibit distinct patterns of cell death in scattered individual heart muscle cells along with viral infection in the endothelium (4) – challenging the view that severe SARS-CoV-2 infection is associated with myocarditis.

Silence of the gene

The *TYW2* gene's epigenetic inactivation in tumors was discovered nearly half a century ago – but only now have researchers discovered its cause… and its consequences. A new study shows that *TYW2* silencing enhances pro-metastatic features and is associated with decreased survival (5).

References

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2. *F Schmidt et al., J Exp Med, e20201181 (2020). PMID: 32577658.*
3. *S Karvinen et al., Front Physiol, 11, 676 (2020). PMID: 32760282.*
4. *SE Fox et al., Circulation, [Epub ahead of print] (2020). PMID: 32689809*
5. *M Rosselló-Tortella et al., Proc Natl Acad Sci USA (2020). PMID: 202003358.*

Why Didn't They Teach This in Med School?

A series on new (and not-so-new) medical terms and diagnoses that most of us (probably) missed in training

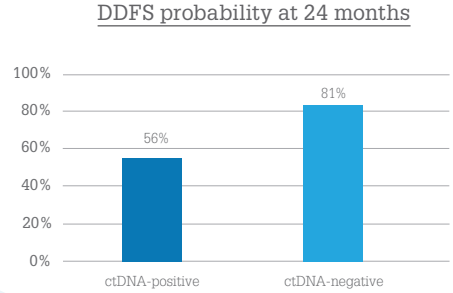
Curated by Ivan Damjanov

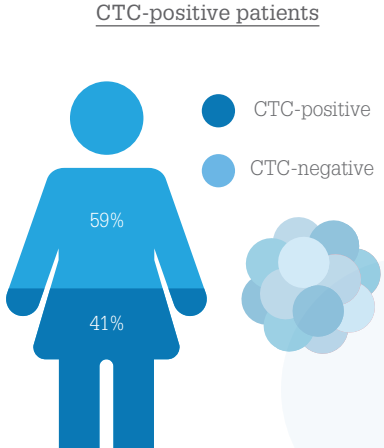
What are serpinopathies?

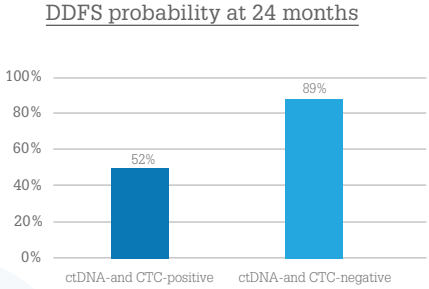
Serpinopathies are a group of hereditary diseases involving the genes that encode one of several serine protease inhibitors, also known as serpins. The largest family of protein inhibitors, serpins include – among others – α1-antitrypsin, α1-antichymotrypsin, C1 inhibitor, and antithrombin.

The best-known serpinopathy is α1-antitrypsin (AAT) deficiency, which is caused by mutations in the *SERPINA1* gene. It has a prevalence of 1 in 2,500.

Abnormal AAT produced by the mutated gene is misfolded and retained in the rough endoplasmic reticulum of liver cells in form of microscopic cytoplasmic globules. Such conformational changes of abnormal proteins are common to all serpinopathies, which are therefore also known as protein conformational disorders, protein misfolding diseases, or simply proteinopathies.

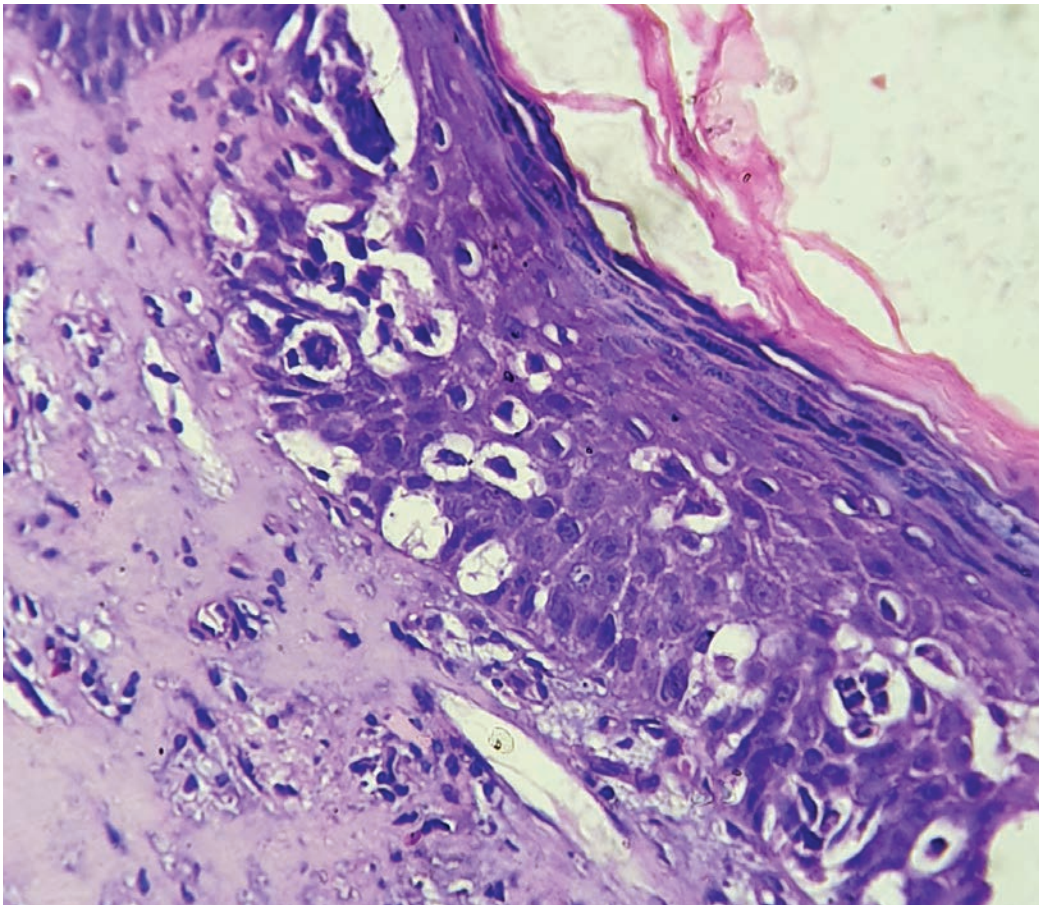






In Focus: Mammary Paget's Disease

A case report of Paget's disease of the breast



A 52-year-old female presented with a well-defined, erythematous, crusted plaque over her left breast for two years. The plaque started as a small, erythematous papule over the areolar area of her left breast and slowly enlarged, extending over the nipple. The left nipple slowly became flattened as the lesion grew and formed a scaling crust (see Figure 1). The patient experienced occasional pain and tingling over the plaque. No underlying mass was detected on clinical examination or on subsequent ultrasound and mammography.

Histopathological examination showed acanthosis without any parakeratosis. There were nests of large, round, pale-staining cells with large, irregular nuclei and prominent nucleoli in the epidermis (see Figure 2). No underlying malignancy was noted. The cells were positive for CK7, CAM 5.2, and HER2, but negative for HMB 45 and CK 5/6. This constellation of clinical and histological features prompted a diagnosis of Paget's disease of the breast.

First described by James Paget in 1874, Paget's disease of the breast is a malignant disease that presents itself as an eroding, bleeding ulcer or eczematous lesion of the nipple (1). Paget's disease is often associated with primary invasive or in situ carcinoma of the breast. Due to clinical overlap with chronic eczematous dermatitis, underdiagnosis or misdiagnosis causes a median delay of six months in starting the proper treatment (2). Treatment and prognosis depend on the presence and invasiveness of underlying malignancy.

References

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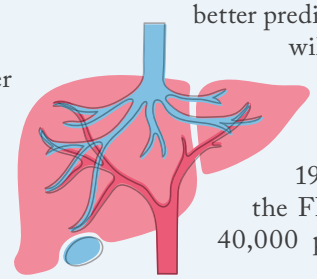
Testing, Testing...

Repeated measurements of the FIB-4 blood biomarker help predict cirrhosis risk

Fat accumulation in the liver can lead to cirrhosis or cancer. Symptoms of liver cirrhosis occur at a late stage when the chance of treatment is low – and, although blood tests can measure liver damage, cirrhosis risk prediction remains a challenge. New research has shown that repeated use of the fibrosis-4 index (FIB-4), a score that evaluates the degree of fibrosis, could better predict whether a patient will develop cirrhosis.

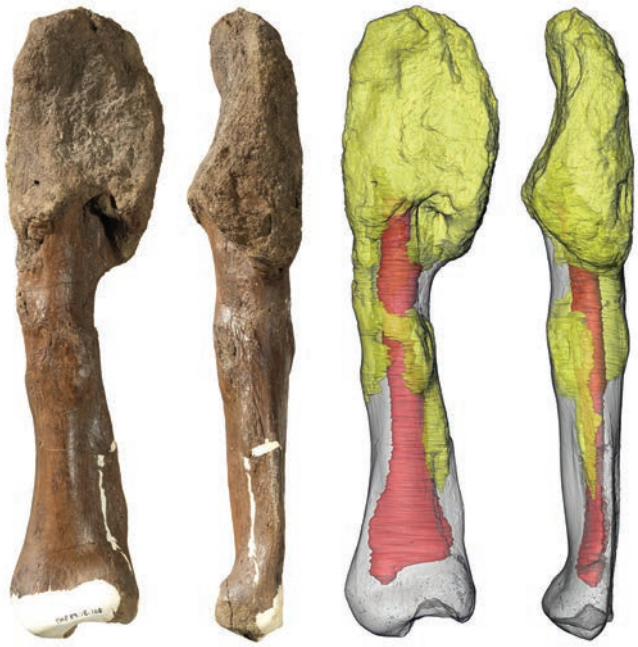


The team surveyed data from between 1985 and 1996, which included the FIB-4 scores of over 40,000 people in Sweden. After identifying those who developed cirrhosis, they found that risk increases in people whose FIB-4 score rises between two tests – and that risk decreases again when it falls. Almost half of those who developed cirrhosis were spotted retrospectively using FIB-4 scores – and future research may improve our ability to detect potential cirrhosis patients.

Reference

1. *H Hagström et al., J Hepatol, [Epub ahead of print]. PMID: 32621944.*

IMAGE OF THE MONTH



Dinosaur Osteosarcoma

Researchers report the first-ever case of osteosarcoma diagnosed in a dinosaur, *Centrosaurus apertus*, by a multidisciplinary team.

Credit: Danielle Dufault/Royal Ontario Museum/McMaster University

Do you have a photo suitable for Image of the Month?
Send it to edit@thepathologist.com

TWEET of the month

"If there was one thing I would want to convey […] it would be that the root of all microscopy is based on an understanding of grossing."

Cory Nash, MS, PA(ASCP)
Read the full Twitter thread at: tp.txp.to/Nash

Lifesaving Lab-on-a-Chip

A new, fully automated chip can type blood in five minutes

In an emergency, do you know what type of blood you would need? Many don't – and there often isn't time to perform accurate blood typing, which requires specialized laboratory equipment and personnel. That's why researchers from Tokyo University of Science have created a fully automated lab-on-a-chip that performs highly sensitive blood typing in just five minutes (1). The chip dilutes the blood, mixes it with air, and homogenizes it before introducing it to four reaction chambers: one each for A, B, and D antigens and a negative control. The user simply loads the blood, starts the chip, and reads the results by observing coagulation in the reaction chambers.

A lifesaver? Perhaps. Senior author Masahiro Motosuke said that the chip "will lead to the simplification of medical care in emergency situations and will greatly reduce costs and the necessary labor on parts of medical staff (2)."

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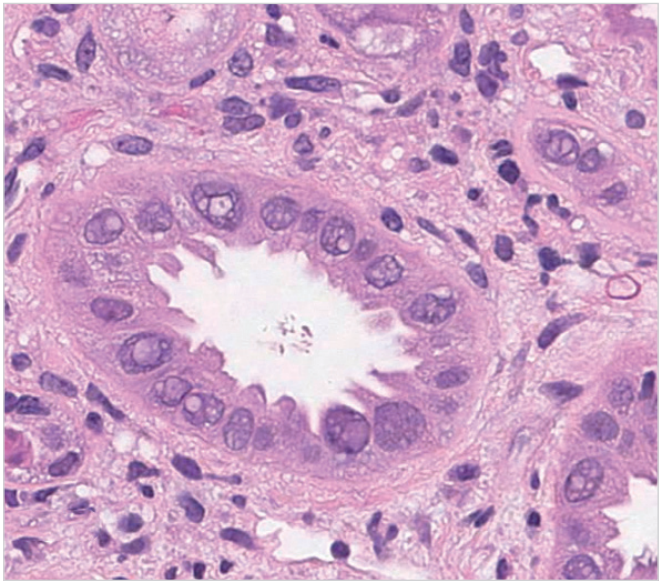
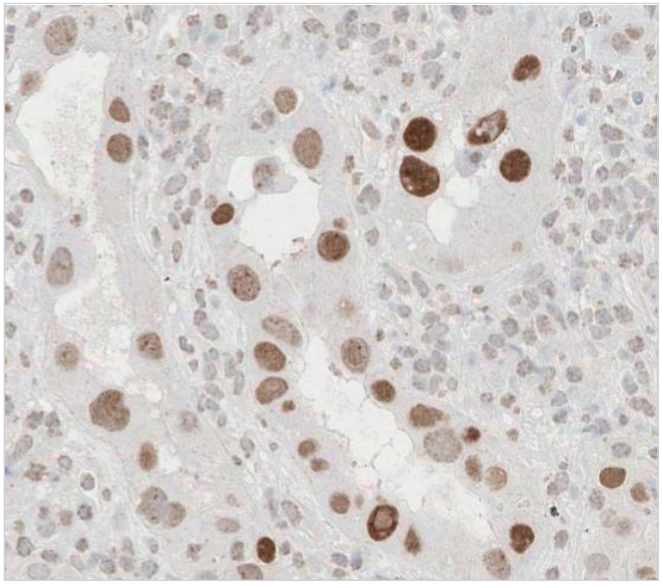
2. *Tokyo University of Science (2020). Available at: https://bit.ly/2QlaZob.*

CASE OF THE MONTH





A five-year-old male with history of autosomal recessive polycystic kidney disease status post-renal transplant presented to his nephrologist with increasing creatinine. Donor serologies were CMV negative, EBV positive; recipient was CMV negative, EBV negative. Due to worsening renal function, a renal biopsy was performed. Representative histologic findings are shown in the images below, including a confirmatory immunohistochemical stain performed with the antibody to simian virus 40 (SV40).

Which of the following is the most likely cause of disease?

a) Epstein Barr virus (EBV)
b) Polyoma BK virus (BKV)
c) Cytomegalovirus (CMV)
d) Herpes simplex virus (HSV)
e) Polyoma JC virus (JCV)

Answer to last issue's Case of the Month…

b) It often lacks high grade histologic features but is still considered grade IV.

This tumor is considered grade IV regardless of the tumor's histologic features; all of these tumors are considered to have a dismal prognosis. Most such tumors appear in the midline, but diagnosis cannot be made without molecular or IHC testing to confirm the mutation.

Case submitted by PathologyOutlines.com. Contributed by Rawia Mubarak Mohamed and Najla Saleh Ben Gashir, Sheikh Khalifa Medical City, Abu Dhabi, United Arab Emirates. Discussion by Maria Martinez-Lage, Massachusetts General Hospital, Boston, Massachusetts, USA.

To register your guess, please go to http://tp.txp.to/0920/case-of-the-month
We will reveal the answer in next month's issue!

Case of the Month is curated by Anamarija M. Perry, University of Michigan, USA.

In advanced ovarian cancer,

If you're not testing for HRD, you're not seeing the whole picture

1 in 2 women with HRD-positive tumors do not have a *BRCA1/2* mutation[1-4]

Homologous recombination repair deficiency (HRD) testing identifies tumor characteristics—beyond *BRCA1/2* mutation—that make it sensitive to PARP inhibition.[1,5]

Personalized medicine begins with personalized pathology. Discuss establishing a testing protocol for HRD in ovarian cancer with the multidisciplinary team at your institution.[6-8]

Learn more at **testforHRD.com**

BRCA, breast cancer susceptibility gene; PARP, poly ADP-ribose polymerase.

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Molecular Pathology

In My View

Experts from across the world share a single strongly held opinion or key idea.

Beyond the Petri Dish

Metagenomic sequencing is poised to revolutionize clinical microbiology

By Todd Dickinson, Founder and Chief Executive Officer of Arc Bio, Cambridge, Massachusetts, USA

Since the mid-1990s, clinical microbiology laboratories have harnessed the power of the genome to develop faster, more sensitive diagnostic tests. Today, molecular tests such as polymerase chain reactions are a routine and valuable part of most labs. Although molecular tests offer significant advantages over more antiquated techniques such as culture, they are still limited to a fairly narrow window of microbes that can be detected in a single sample. This means that labs may need to perform multiple blood draws from a single patient to sequentially test using several assays. And to decide what test to use and when, laboratorians must also piece together different kinds of information, including the patient's clinical history, differential diagnosis, and local epidemiology. All of these steps can add cost, delay results, and place an unnecessary burden on the patient.

Metagenomics offers the next leap forward for infectious disease diagnostics by providing a powerful tool that can detect potentially unlimited numbers of known and novel microbes from a single sample in one test. Shotgun metagenomic sequencing enables users to easily evaluate the diversity of entire microbial populations, including novel, emerging, or uncharacterized pathogens. For the clinical microbiologist, this means the potential for truly hypothesis-free detection and quantification of all microbes in a patient sample without the need for any prior culture or growth steps.

Because of its impressive diagnostic yield, shotgun metagenomics can also rapidly provide information on antimicrobial resistance genes carried by pathogens – and even human genetic information, such as host immune response – from a single sample. Importantly, it also has the potential to be deployed in early sentinel programs that can alert health systems to the emergence and transmission of novel pathogens. Never has the importance of sensitive, unbiased surveillance tools been more evident than during the COVID-19 pandemic. The earlier we can detect a problem, the more effectively we can respond.

Despite its potential, broad adoption of metagenomics hinges on our ability to clear away some of the key barriers that face clinical microbiologists today. The technical and resource demands of setting up shotgun metagenomics in the microbiology lab can be significant.

"Despite its potential, broad adoption of metagenomics hinges on our ability to clear away the key barriers."

Developing and deploying turnkey solutions that empower laboratorians to quickly and cost-effectively integrate metagenomics into their workflow are a must. Once microbiologists have these tools

in hand, it is critical that we also provide evidence-based guidelines and protocols for validation and implementation, as well as clinical utility data to help guide testing in populations that will receive the most benefit. Labs are making headway toward this goal, as we demonstrated recently in a joint publication from scientists at Arc Bio and Stanford's Clinical Virology Laboratory (1). Our research compared a turnkey shotgun metagenomics platform to gold standard qPCR methods and found similar performance in terms of sensitivity, limit of detection, and viral quantification. However, unlike single-plex PCR or even syndromic PCR panels, metagenomics can interrogate a single sample for potentially thousands of microbial species and strains in a single analysis. This translates to impressively high diagnostic yield from a single blood draw. For patients where coinfections are clinically relevant, metagenomics can provide a powerful diagnostic tool to help inform and guide care. Although it remains early days, metagenomics is gaining momentum quickly and, in my view has the potential to become the new gold standard in infectious disease diagnosis and management.

Reference

1. *ML Carpenter et al., "Metagenomic next-generation sequencing for identification and quantitation of transplant-related DNA viruses", J Clin Microbiol, 57, e01113-19 (2019). PMID: 31554674.*

Pathologist–AI Unity

To enhance the work of pathologists, artificial intelligence must be responsive to their needs

By Andrew Schaumberg, Research Fellow at Harvard Medical School, Boston, Massachusetts, USA

Pathologists across the world frequently share patient cases on social media to get input from peers. James Nix and colleagues found that pathologists in developing countries often use social media, and 22 percent of the posts analyzed were seeking opinions on diagnosis (1). But social media does more than bring subspecialty expertise to hospitals where it may not otherwise be available. Social media increases access to pathologists and other physicians worldwide, potentially improving patient care – in line with the United Nations' Sustainable Development Goal #3: good health and wellbeing (2).

"Given H&E photomicrographs of a patient case and a brief text description, pathobot finds similar patient cases on social media and PubMed and predicts the disease state of a case."

But how can we make social media work better for pathologists? We've developed a new tool, called "pathobot," to help (3). Given H&E photomicrographs of a patient case and a brief text description, pathobot finds similar patient cases on social media and PubMed and predicts the disease state of a case (nontumor/infection, benign/low-grade, or malignant) (4). The synergistic relationship between artificial intelligence (AI) and responsive pathologists is crucial for pathobot's success. When a pathologist on Twitter mentions "@pathobot," other pathologists can see the tool work – and, in some cases, they are inspired to share and collaborate with us (5,6). With more case data, pathobot's AI improves and its search database expands to cover more disease entities. Even better, pathobot can be used repeatedly as pathologists further characterize a specific case – including differentials, immunohistochemistry, additional H&E photomicrographs, and more (4).

In AI, we often regard data as a static

"In AI, we often regard data as a static corpus – but we must remember that these data come from pathologists who are living, breathing, caring people."

corpus – but we must remember that these data come from pathologists who are living, breathing, caring people. Pathologists absolutely want to share and discuss cases on social media – and that's precisely where AI can help. Specifically, AI can do the "grunt work" to find similar cases, find key pathologists worldwide with similar cases, bring pathologists together to discuss the next steps in a patient's care, adapt search results as more information becomes available, and grow as more pathologists collaborate with us. Our approach has been so well-received that we won the #PathVisions2019 Poster Award for Best Image Analysis (5,7).

But it's not enough to simply offer a tool for pathologists who are already engaged to use. To reach the greatest possible number of pathologists and patients, let's get a smartphone on every microscope. Why a smartphone? With the advent of COVID-19 and the concomitant increase in telepathology, sharing photomicrographs and teleconferencing via smartphone is more common than ever. Additionally, residents may not have access to microscope-mounted cameras, but still wish to share cases, so an inexpensive way to mount a smartphone to a microscope may help with education.

To this end, we have begun to address two main challenges:

- How can one cheaply mount a smartphone to a microscope? To reduce costs, we 3D print a system of parts called #pathobox (8), which we ship for free through our organization, @pathobotology (9). (We appreciate donations, particularly from well-resourced institutions or crowdfunding. Currently, I fund these efforts personally, with support from Mariam Aly and family.)
- How can one cheaply approximate a whole-slide image from the limited field of view a smartphone and microscope offer? Whole-slide images are important in digital pathology, at least in part because these images provide more context than a single field of view at a microscope. Unfortunately, whole-slide image scanners may be prohibitively expensive for low-resource regions or hospitals, and whole-slide images are far too large to post on social media (10,11). Therefore, we freely provide the #pathopan tool (12), which stitches together overlapping photomicrographs to form a small, low-quality whole-slide image – similar to the way a smartphone stitches together overlapping photos to form a panorama.

To improve the pathobot AI and ask increasingly sophisticated questions of our data, we are always looking to collaborate more widely with pathologists. Our goal? To leave tedious work – like manufacturing smartphone mounts and matching new cases to similar ones in our archives – to the robots and devote ourselves to the challenges only we can take on. It takes a human to collaborate with colleagues, to address evolving health challenges, and – most important of all – to care for a patient.

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Critical Connections

Staying connected is vital in unprecedented times

By E. Blair Holladay

2020 looks nothing like we expected. As we enter the last quarter of the year and turn our thoughts to 2021, the uncertainties we face far outweigh any clarity we may have.

One thing, however, has become crystal clear over the past six months: that staying connected is essential to both our professional and our personal lives. Since March, when organizations – including the ASCP – started sending employees home to work remotely, we have launched an array of new initiatives to keep our members connected and informed while maintaining social distance to stop the spread of COVID-19.

From Virtual Grand Rounds to regular Town Hall events on topics important to pathologists and medical laboratory scientists, we've launched a variety of efforts to connect. In our podcasts and virtual meetings, subject matter experts from across the country have discussed diagnostic versus serology testing, public policy opportunities, health disparities, and much more. As the pandemic persists, we will continue to bring these events to the community, with national experts providing insight and knowledge on how we can move forward – and hopefully move past – these challenging times.

When the pandemic started, we knew we had a duty to provide the essential information pathologists and medical laboratory scientists would need to inform their work and provide better patient care. As the months have passed, we've added to that mission, helping to stabilize an increasingly moving target as we learn more about SARS-CoV-2.

Most importantly, we knew it was imperative to stay connected to our profession because those connections are what help people push through the toughest and most uncertain times. Research proves that there are myriad health benefits to staying personally connected – improving your immune system, lowering stress levels, and ultimately lengthening lifespan. Staying professionally connected also has enormous benefits. It deepens your sense of community – and right now, entrenched in a global fight, the pathology and laboratory community is stronger than ever. With every one of us is doing our part to provide necessary testing and essential patient care, that community gives us colleagues to lean on when we come across something novel or unexpected. The ability to reach out to colleagues for advice, whether in person or virtually, has been of paramount importance during the pandemic. Keeping connections alive and thriving, whether between two people or among groups of thousands, strengthens the foundation of pathology and laboratory medicine – the bedrock of healthcare.

"Research proves that there are myriad health benefits to staying personally connected."

We don't know what we will face in the last few months of 2020 or what's around the corner in the new year. But we know that together we will endure. Together, we will thrive. Together, we will achieve more than we thought possible. And together, we will help bring this world back to a healthier place.

THINK OF THE CHILDREN

Two experts in childhood cancers give their take on the intricacies of pediatric laboratory medicine, how molecular techniques help, and where they think the field will go next.

Cancer diagnostics and treatment have come a long way over the past 50 years. Before the advent of chemotherapy, surgery – sometimes aided by radiation therapy – was the only effective intervention for cancer patients. Although this approach has long been the primary treatment modality for adults, where the goal is to remove the entire tumor, surgery is not as effective in younger patients. Childhood cancers, even those with apparent similarities, are distinct from those seen in adults – with different developmental stages, tissues of origin, mutations, and gene fusion events.

With the discovery by Sidney Farber that a folate antagonist improved survival in children with acute lymphoblastic leukemia came new hope in the fight against pediatric cancers. The application of chemotherapeutic agents quickly spread across all childhood tumors – and it became clear that children typically respond better to chemotherapy than their adult counterparts. Survival rates for pediatric cancer patients – which until this point had been far inferior to adults – began to rise and even surpassed those for adult cancers.

More recently, the field of immuno-oncology has begun to flourish – but, despite showing promising signs in the adult cancer world, immunotherapy is less effective in pediatric cancers. The efficacy of immunotherapy depends on the strength of the patient's own immune response – often absent in childhood cancer patients with few tumor mutations. More encouraging is the use of therapies targeting unique gene fusion events. These events are common in pediatric cancers, clearly differentiating their molecular pathogenesis from that of adult disease.

As precision medicine continues to serve patients of all ages, it is clear that our understanding of the genetic drivers behind childhood cancers will be key to future progress and more finely tuned therapeutic interventions. Two initiatives aiming to improve outcomes for childhood cancer patients are the OncoKids Cancer Panel and the Pediatric Cancer Genome Project. We spoke to two of the experts working at the forefront of pediatric laboratory medicine to learn more about the cutting-edge molecular approaches to childhood cancer diagnosis and treatment.

A WORLD APART

Childhood and adult cancer drivers are distinctly different – and the approaches must be equally specialized

An interview with Tim Triche

What led you to pediatric laboratory medicine?

I originally trained in surgical pathology, but I was also interested in research and chose to do a National Cancer Institute (NCI) fellowship at the National Institutes of Health. I thought this would be a brief stint and then I would move on – but, once I arrived, I discovered that they had no full-time pediatric pathologists. I was asked to present tumor boards and review cases with the pediatric oncologists and surgeons, which started as a sideline, but soon became my main interest. There was such a wealth of interesting cases arriving at the NCI that, eventually, I focused exclusively on pediatric cases. Although I still have an interest in adult cancer, the vast majority of my time for the last few decades has been spent on pediatric oncology, and I have expanded from surgical pathology into many other areas.

Tell us about the OncoKids Cancer Panel

While working at the NCI, it quickly became obvious that the adult and pediatric cancer cases I signed out side by side were very different from each other – not just in terms of the diagnoses, but also their characteristics; for example, where tumors occurred and how they behaved. Why were they so different? We didn't know at the time that the drivers of pediatric cancer are completely different from those of adult cancers. There is very little overlap because most adult cancers are the result of accumulated mutations in the genome; however, we knew early on that this wasn't the case for childhood cancers. We started to search for features that could explain pediatric cancers and noticed recurring chromosomal breaks across various tumor types. We realized that these breaks – where a piece of one chromosome fuses with another – were likely giving rise to driver genes, and we eventually characterized them as fusion genes. These fusion genes are extremely common in childhood cancer and, although they sometimes occur as a secondary issue in adult cancer, they are frequently a primary feature in pediatric cases.

When the NCI-MATCH (Molecular Analysis for Therapy Choice) Trial was announced in 2015 to assess the efficacy of targeted therapy for patients with specific gene mutations, I realized that it would miss most of the important features

of pediatric tumors. The main types of cancer in adults are lung, colon, breast, and prostate – all carcinomas that arise from lining or covering tissue. In contrast, childhood cancers ultimately derive from the mesoderm or neurectoderm; there is essentially no carcinoma. I wanted to create a cancer assay that reflected the tumors that occur in children, adolescents, and young adults, so we at Children's Hospital Los Angeles (CHLA) spent several years developing the OncoKids panel with Thermo Fisher Scientific. Working with pathologists, oncologists, surgeons, and industry experts, we assembled the content required for pediatric cancers. Although some of the assay's DNA content remained the same due to an overlap between mutations in children and adults, the RNA content is unique. There are approximately 1,400 different combinations of potential fusion genes in childhood cancers, which we reflected in a panel heavily skewed toward RNA content.

"Although I still have an interest in adult cancer, the vast majority of my time for the last few decades has been spent on pediatric oncology, and I have expanded from surgical pathology into many other areas."

How is it used?

The panel is particularly useful because it can i) identify inherited components, which account for around 15 percent of childhood tumors; ii) establish a precise diagnosis, which is crucial when introducing potentially toxic chemotherapeutic agents; and iii) detect specific mutations that can be treated with targeted therapeutics. For example, relatively early on in our use of the panel, a very young patient presented at CHLA with a life-threatening neck tumor – but the original biopsy interpretation was inconclusive. We were asked to carry out a rapid analysis using a small amount of formalin-fixed, paraffin-embedded tissue, which is historically difficult to assess. We processed the tumor material and identified an unusual childhood cancer gene fusion – tropomyosin fused to tyrosine kinase – providing us with a target for therapy. Working with the head of our solid tumor service, we deployed an NTRK inhibitor that same day, and the patient responded very favorably. The tumor became undetectable over a period of weeks and, with no further treatment, the patient has remained disease-free for two years. This case demonstrates the importance of knowing exactly what you're treating and selecting the appropriate agent.

The RNA content in the panel is crucial because gene fusions often establish an unequivocal diagnosis and determine how the patient will be treated and whether there is a suitable targeted agent available. We now present these results in three different weekly or biweekly tumor boards: one for brain tumors, a second for leukemia and lymphoma, and a third for solid tumors. The molecular pathologists discuss the findings from the OncoKids panel in conjunction with the surgical pathologists – and that comprehensive diagnostic workup is then discussed with the treating oncologists.

What are the main differences between approaches for children and adults?

Before the advent of chemotherapy, the only effective treatment for cancer was surgery. The survival rate for childhood cancer in that era was abysmal because surgery rarely works by itself in these cases; pediatric tumors tend to move around and often become metastatic early in the course of disease. However, the outlook changed with Sidney Farber's discovery that drugs could cure leukemia. That knowledge rapidly translated to all other childhood malignancies – and it was quickly established that children respond far better to chemotherapy than adults. Treatment regimens in children quickly became multimodal affairs whereby surgical excision was followed by chemotherapy and radiotherapy – and survival rates improved drastically.

Another key difference is the effectiveness of immunotherapy, which relies on the development of a host immune response that the tumor suppresses. Although adults often respond well to immunotherapy, childhood cancers aren't usually associated with tumor burden and tumor mutations. As a result, immune response in childhood cancers is nowhere near as robust, and immunotherapy is reserved for rare pediatric cases with a significant amount of mutation. That leaves us still very much dependent on classic chemotherapeutic agents – but our hope is that we will see an increase in less toxic agents that target the gene fusions common in childhood cancer.

How has COVID-19 affected your work?

Many of the sensitive next-generation sequencing (NGS) technologies that we are accustomed to using for pediatric cancer suddenly became very useful, especially when dealing with poor-quality nasopharyngeal swab specimens to detect the presence of SARS-CoV-2. Specifically, we have been able to sequence viral isolates to determine whether infected individuals have transmitted the virus between themselves or acquired it separately in the outside community. It is through this testing that we spotted a viral strain called D614G, a variant of COVID-19 that was detected in Europe and spread to the east and west coast of the US. It's not uncommon to see multiple slight sequence variants from a single isolate – and this issue of SARS-CoV-2 genomic stability is both fascinating and crucial for the development of any potential vaccine. We will continue to monitor these variants for any changes in the amino acid sequence that could, in turn, alter the spike protein and affect antigenicity.

Although the severity of COVID-19 was originally assumed to be worst in adults, the reality is that children – particularly the very young – can also have an extraordinarily severe response. In some pediatric cases of infection by SARS-CoV-2, we see a unique host autoimmune response that results in cytokine storm and widespread tissue damage, producing rashes on the skin and, in some cases, multiple organ failure. There are many hypotheses as to why we only see this response in children. My own suspicion is that many older patients have mild or asymptomatic disease because they already possess partial immunity and can rally old memory B or T cells to fight infection. In contrast, young children are being exposed to these viruses for the first time – and, although most immune systems can produce an appropriate response, some launch a destructive response that is not attuned to the viral infection. We still need to learn a lot more about this type of autoimmune disease and the potential to treat these young patients with cytokine inhibitors.

"The RNA content in the panel is crucial because gene fusions often establish an unequivocal diagnosis and determine how the patient will be treated and whether there is a suitable targeted agent available."

How has pediatric medicine changed over the course of your career?

I distinctly remember seeing young leukemia patients and realizing that I would need to have a very difficult conversation with the parents about the awful prospects their child faced. In those days, leukemia was largely incurable and the mortality rate was nearly 100 percent. However, over the course of a decade after the arrival of chemotherapy, we flipped to having an almost 100 percent survival rate – a phenomenal change in the outlook for childhood cancer patients.

Traditionally, clinical presentation is a cluster of symptoms and findings that we label and treat accordingly. If there is one overriding theme over the course of my career, it's that we've come to appreciate the value of personalized medicine. Every patient possesses unique features against which we can develop finely tuned therapeutic interventions and management. And that's one of the major reasons for our improved outcomes; we now recognize categories, understand genetic drivers, and instigate patient- and disease-specific treatments. We've also learned that, although pediatric cancer types can occur in patients aged two or 22, the behavior of those cancers will be extremely different. We now take into account age, the specific disease evolution, and any polygenic influences, all of which provide pediatric medicine with the opportunity for precise diagnosis and treatment.

Where will the field go over the next 10 years?

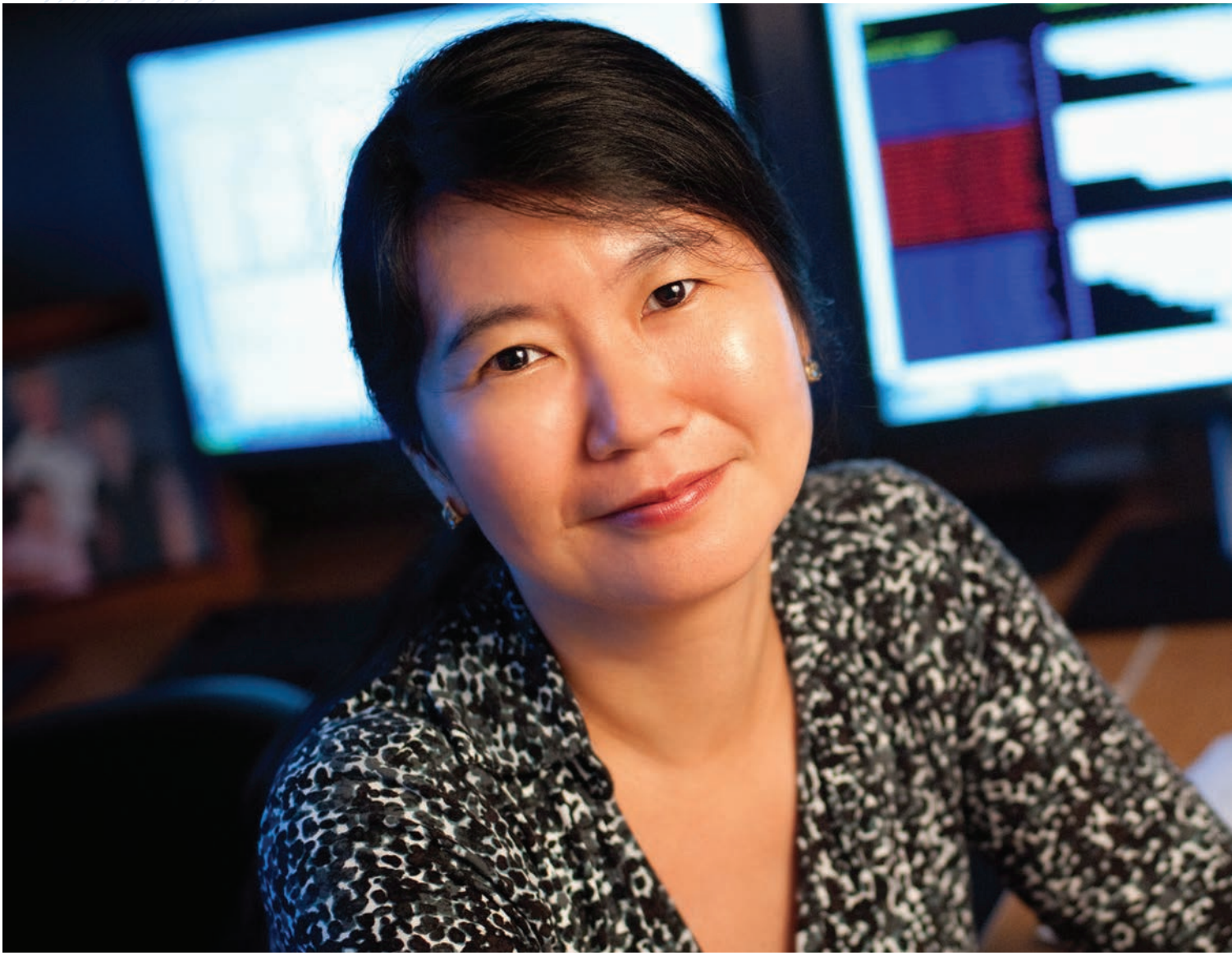
The personalized approach will become increasingly evident as the precision and accuracy of our tools improve. There are clearly many questions to be answered – and, although it seems as though every answer begets 10 new questions, we're also developing the tools to analyze more and more data, variables, and features. Some interesting possibilities are emerging, especially in terms of tools that aggregate vast amounts of data beyond the capability of any one person. Machine learning and artificial intelligence now facilitate worldwide databases containing extensive information about all kinds of unique cases. We can use these databases to compare and contrast patients' features with cases across the globe, ensuring we make informed decisions about diagnosis, treatment, and management.

As this becomes increasingly widespread, we'll be spending more time in front of our computers trying to make sense of it all. I think these tools will become dominant features in medical management for patients of all types in the future – especially because that intelligence database can help us understand the results of our increasingly complex diagnostic assays and workups. As a result, to thrive in pediatric – and all – medicine in the future, you will have to feel comfortable working with your computer and quantitative analytic methods.

What advice would you give to anyone considering a career in pediatric medicine?

Anyone entering the field today will benefit enormously from basic training in math and statistics so that they can appreciate the use of new tools and draw conclusions from them. Because I wanted to become a particle physicist early on in my own career, I developed an appreciation for analytic methods and mathematical analysis. I never really envisioned these skills being useful in my journey through pediatric laboratory medicine – but, as the analytical tools in our armory grew, it became increasingly obvious that quantitative analysis is crucial for interpreting data that directly relates to a patient's diagnosis. For example, we often work with biomarker profiles that provide multiple variables, which can be extremely powerful predictors if you are comfortable aggregating and analyzing the important features.

It's also important to understand the limits, as well as the capabilities, of analytic methods. Laboratory values are frequently numeric and you have to decide when certain values are outside the normal range. In my opinion, virtually every aspect of pathology and laboratory medicine is ultimately influenced by the need to understand and use quantitative analytic methods. My advice to those considering a career in this space would be to develop these skills as part of your overall medical training.



STRENGTH IN NUMBERS

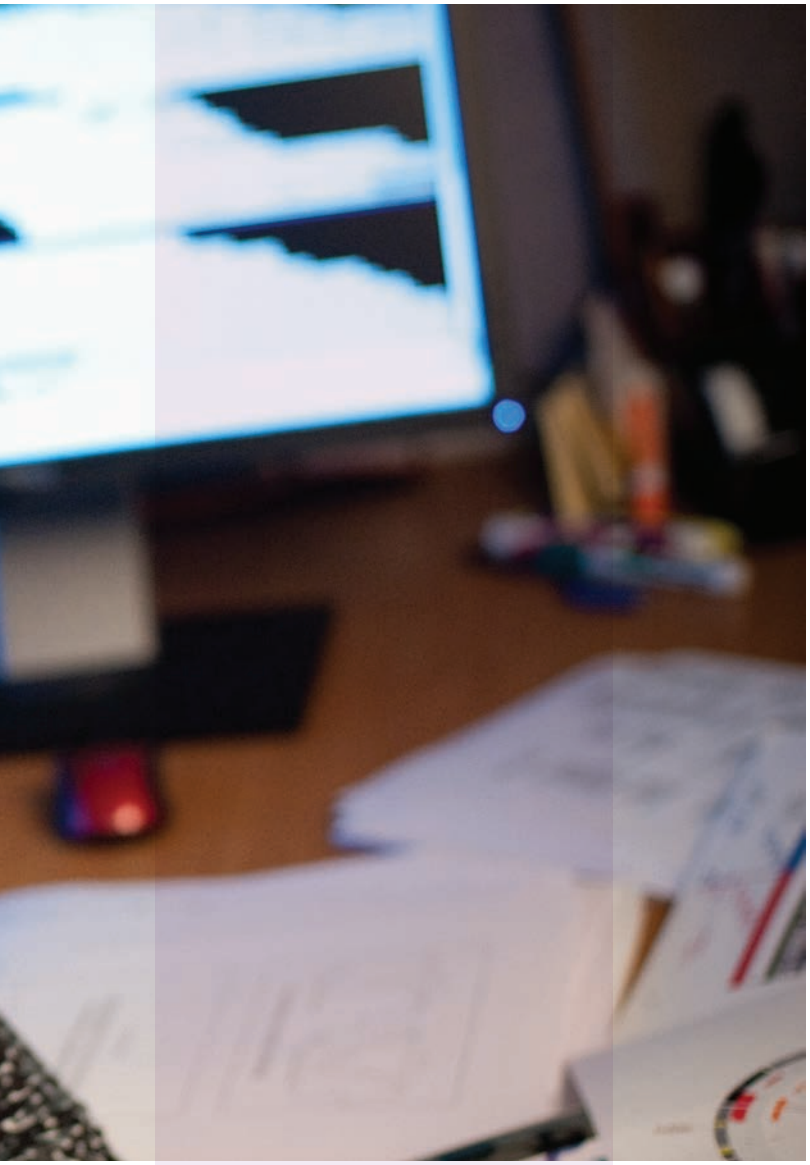
Data sharing is critical to gathering detailed information on rare pediatric cancers

An interview with Jinghui Zhang

How did you get into pediatric laboratory medicine?

I joined St. Jude Children's Research Hospital in 2010 after working at the NCI. At the time, The Cancer Genome Atlas Program was the center of attention – everyone seemed to be working on this project focused on adult cancer. In 2010, St. Jude invested US$65 million in the Pediatric Cancer Genome Project, created in collaboration with Washington University School of Medicine, to sequence the paired tumor-normal genomes of 600 pediatric cancer patients. Its goal was to define the genomic landscapes of some of the least understood and most challenging childhood cancers. And it was one of the main reasons I transitioned from the adult cancer world to pediatric cancer.

Another of my motivations stemmed from the 20 years of genomic data I had been studying throughout my career. When I was at the NCI, I analyzed a subset of pediatric leukemia patients and discovered an activating mutation in a kinase



gene called *JAK2*. We started discussing how to apply this finding to clinical trials to discover JAK inhibitors that could potentially treat patients – and that was the moment I began thinking about working in pediatric oncology. I thought, "What could be more rewarding than research that leads to improved clinical outcomes in childhood cancer patients?"

What is the value of precision medicine for pediatric cancers?

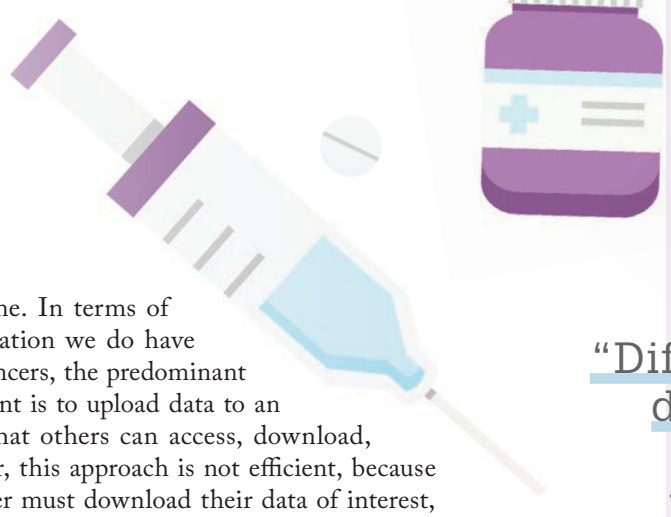
Precision medicine has existed for a relatively long time in the context of pediatric cancer; however, in the past, we used techniques such as cytogenetics and polymerase chain reaction testing to find biomarkers and classify cancer subtypes. With next-generation sequencing (NGS), we can classify patients much more precisely and effectively. For example, in pediatric leukemia, we can look at gene fusion patterns to define low- or high-risk patients and apply chemotherapy according to their global genomic profile. Another great benefit of precision medicine is the range of treatment options we can offer patients with specific mutations. We can search for genes such as *JAK2* and apply targeted therapy if there are activating mutations or gene fusions in the kinase. For patients with vulnerabilities, such as high mutational burden or DNA mismatch repair, NGS can assess potential response to immunotherapy – and, in solid tumors, we can ask whether the patient is susceptible to PARP inhibitors.

"We can also monitor patients for potential secondary cancers further down the line, and potentially intervene with preventative procedures to decrease the chance of recurrence during adulthood."

But the aspect of precision medicine that is most specific to the field of pediatrics is cancer susceptibility. In 2015, we performed a comprehensive analysis of germline inherited susceptibility to pediatric cancer because of the uniqueness of our patients with cancer at a very young age. We can discover pathogenic mutations in the germline genomes of pediatric cancer patients – and include family studies to analyze inheritance patterns between relatives. We can also monitor patients for potential secondary cancers further down the line, and potentially intervene with preventative procedures to decrease the chance of recurrence during adulthood.

What is St. Jude Cloud and why is it so important?

A really challenging aspect of working with pediatric cancers is that they are such rare diseases. For certain subtypes, we sometimes find that there are fewer than 200 cases in the USA, which often means insufficient access to samples and not robust correlative analyses between genomic profile



and clinical outcome. In terms of sharing the information we do have on rare pediatric cancers, the predominant model at the moment is to upload data to an online repository that others can access, download, and share. However, this approach is not efficient, because every individual user must download their data of interest, which requires resources and time. It's also not sustainable for institutions or labs that don't have a large digital infrastructure, because the data takes up so much local space. Even for those of us with access to a high-throughput computing facility and professional support from computer scientists and software engineers, this can still be a time-consuming and difficult task.

And so, we decided to create a cloud-based infrastructure that provides users with a platform on which to upload and share data without having to download anything locally. We launched St. Jude Cloud in 2018, and the data on the platform has already been accessed by 2,500 users weekly and more than 200 researchers from 80 institutions worldwide have been granted access to raw genomic data. Although we haven't reached the point at which all analyses can be conducted on the cloud, we believe the future lies in working entirely with cloud-based data – and that's what we're now working toward. This ability will be particularly useful for laboratories and institutions with limited computational resources; they will be able to integrate their own data with those already available on St. Jude Cloud and tap into this wealth of information.

The efficacy of this approach is clear – even within the first year of St. Jude Cloud, we had more users access our data than in the previous five years of uploading data to the public repository. Our users include experienced labs using the data to find new diagnostic markers, develop specific immunotherapies for pediatric cancers, investigate whether drugs effective in adult cancer can be applied to childhood cases, and even search for new cancer susceptibility genes in germline genomes. We are in the process of preparing a manuscript to demonstrate how useful the cloud-based platform can be for discovery.

Tell us more about the Pediatric Cancer Genome Project…

The initiative was introduced in 2010 by James Downing, CEO of St. Jude Children's Research Hospital, to generate high-quality NGS data from the genome. It was important to focus on the whole genome – rather than the exome that only targets the gene-coding regions – because a high proportion of pediatric cancers are driven by gene fusions or DNA rearrangement events. Many of these DNA aberrations occur in noncoding regions, making them impossible to discover by targeting the exome alone. In all, we performed whole-genome sequencing for more than 700 paired tumor-normal genomes – and we've been able to identify various novel gene rearrangements. For example, we have discovered the *RELA* gene fusion in patients with a certain type of ependymoma and targetable *NTRK* fusions in a subgroup of high-grade glioma. We put a strong emphasis on studying both the germline and tumor genomes, which has allowed us to discover tumor subtypes with a high mutational burden.

> "Different countries have different regulations – and that's something we're trying to tackle by engaging our global partners and collaborators in pediatric cancer."

Again, we felt that sharing the data St. Jude generated from the Pediatric Cancer Genome Project was crucial, not least because it was valuable to researchers around the world who are working toward improving the care of pediatric cancer patients. We made a conscious effort to publicly distribute the information, uploading the genomic data to the National Center for Biotechnology Information's database of Genotypes and Phenotypes, and the European Genome-phenome Archive prior to publication of all studies related to the Pediatric Cancer Genome Project.

Why was it important for such a project to focus solely on children?

When you compare disease types between children and adults, you find that the most common adult cancers – breast cancer, lung cancer, and prostate cancer – almost never occur in a pediatric setting. Some of the key insights gained from adult cancers may not be applicable for pediatric cancer. Even for cancers that share the same tissue of origin, the driver mutations can be distinct between the two. For example, in many adult brain tumors, *EGFR* amplification is common – but, in childhood brain tumors, it's rare; instead, we often see amplification of *PDGFR* mutations. Notably, the epigenetic regulation caused by histone H3 mutations is present in over

80 percent of pediatric cancers – but completely absent in adult glioblastoma.

In a 2018 study, we compared all of the different mutations in childhood cancer subtypes in a pan-pediatric cancer study with those found in pan-adult cancer studies (1). We found that half of all driver genes in pediatric cancer are not present in adult cancer. Most are involved in transcription regulation caused by gene fusion events unique to childhood cancers. This suggests that you cannot always treat a childhood cancer patient like a small adult, because the molecular pathogenesis is so different. Instead, we must study pediatric cancer independently to search for newer, more relevant targets – and that's something the Pediatric Cancer Genome Project strives to facilitate.

What were the project's most exciting findings?

You'll often hear people comment on the low mutation burden of pediatric cancer. One of the big revelations of the Pediatric Cancer Genome Project is that, even though these cancers are very rare, they are highly heterogeneous and exhibit a wide spectrum of mutation burden. For example, there are diseases like infant acute lymphoblastic leukemia, low grade glioma, and retinoblastoma that only have one driver mutation, whereas others, such as high-grade glioma, have mismatch repair defects that lead to even higher mutation burdens than equivalent adult cancers.

The second big discovery was the importance of gene rearrangements, how often they occur, and how frequently they function as the driver variants in pediatric cancer. We were surprised to find that missense mutations were not the main cause of childhood cancers; instead, up to 60–70 percent of drivers were caused by rearrangements or copy-number alterations. The final thing that struck us was the interplay between germline and somatic cells. There are cases in which pathologists will classify a cancer according to its morphology – but the tumor genome doesn't reveal anything striking. In these cases, it's important to take a deep look at the germline DNA to find out whether there are pathogenic mutations in the germline, some of which may have a second hit in the tumor genome.

How would you describe the impact of the Pediatric Cancer Genome Project?

The US Food and Drug Administration recently released a "Relevant Pediatric Molecular Target List" (2) – and many new targets were based on genomic abnormality discovered by the Pediatric Cancer Genome Project. These targets will allow people to pursue new drugs or test the relevance of existing therapies in the context of childhood cancers. In line with our strong data-sharing vision, we always seek to collaborate, with a view to integrating information and performing robust analysis using extended cohorts. We have participated in a new data-sharing initiative launched by the National Cancer Institute – the Childhood Cancer Data Initiative – which will collect, analyze, and share data to advance the treatment for cancer in children and young adults. Such investments allow researchers to harness collective data and resources to advance pediatric cancer research.

What are your hopes for the field?

A significant challenge we need to address is how to share data not just across the USA, but globally. Different countries have different regulations – and that's something we're trying to tackle by engaging our global partners and collaborators in pediatric cancer. We are working toward a system that follows global data-sharing regulations, but also allows scientists to integrate their own data on the fly, which is especially useful for the rarer cancers.

Such structure is key to the future of the field – and we are investigating the feasibility of a federated data system that would connect constituent databases around the world via a computer network, allowing someone interested in a specific cancer subtype to access and assemble relevant cases from data in many locations. As researchers, this is not a problem we can solve alone. I hope that we can work with technology companies and cloud providers to enable integrated data sharing across clouds in different regions.

My biggest hope is that researchers who are interested in finding cures for pediatric cancers can collaborate and share data across the world. This kind of engagement is so crucial – especially in this field – because no country can fight the battle against pediatric cancer alone. I believe that the COVID-19 pandemic underlines the importance of global collaboration – it's also our greatest weapon in the fight against cancer in children.

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A New Dawn for Comprehensive Genomic Profiling

Published results from early access program demonstrate the reproducibility and reliability of TruSight™ Oncology 500

The more we learn about the complex molecular pathology of different cancers, the more powerful comprehensive genomic profiling (CGP) becomes. Using next-generation sequencing (NGS) to identify genetic alterations that drive cancer, CGP simultaneously examines multiple biomarkers that are included in guidelines and clinical trials, reducing both tissue and time requirements compared to sequential testing methods. An important genomic signature covered by the panel is microsatellite instability (MSI) – an inactivation of mismatch repair genes that prevents the correction of DNA replication errors – which was the first pan-cancer signature approved by the US Food and Drug Administration (FDA). Additionally, coverage for tumor mutational burden (TMB), the recently FDA approved immuno-oncology genomic signature, can be used to estimate the effectiveness of immune checkpoint inhibitor therapy (1).

Both of these genomic signatures, in addition to DNA and RNA variants reveal important information about tumor heterogeneity. TruSight™ Oncology 500 (TSO500), research use only (RUO) assay, analyzes hundreds of these cancer-related genes across 1.94 MB of genomic content using sophisticated software algorithms. Launched in 2019, TSO500 was tested by 13 leading European cancer centers in an early access program (2). Data recently published by the University of Birmingham and Radboud University Medical Center Nijmegen returned particularly promising results (3, 4). We spoke to Andrew Beggs from Birmingham and Leonie Kroeze from Radboud Nijmegen to learn more.

What were the main findings of your recent publication?

Andrew Beggs: We used the TSO500 panel to carry out comprehensive molecular profiling of cancers and compared the results with those from whole-genome sequencing (WGS). The panel was as accurate as WGS and orthogonal techniques at measuring TMB, MSI, single-nucleotide variants, indels, copy number/structural variation, and gene fusions. One of the main benefits of the TSO500 is that it is less expensive than WGS. This lower cost makes it more feasible to complete mass genomic profiling and means that you could theoretically use it for every patient who presents with cancer as a "one-stop shop" for cancer profiling. We also found that the deep sequencing on a targeted panel facilitated a better understanding of tumor heterogeneity and detected rare variants that might otherwise have been missed.

Leonie Kroeze: By using a large sequencing panel, such as the TSO500, we can analyze many biomarkers which will be important for diagnosis and therapy decisions using a limited amount of material. One of the major advantages of the TSO500 is that it includes unique molecular identifiers, which show how many unique DNA molecules have been sequenced. This feature is particularly important to judge the reliability of the detected DNA variants when the DNA quantity is low.

We especially focused on the reproducibility of TMB and MSI values, because both are relatively new NGS-based biomarkers important for predicting response to immunotherapy. After repeating a sample in 10 different sequencing runs, we obtained highly reproducible values. More importantly, the results from 11 different labs across several countries were comparable; interlaboratory reproducibility is crucial if we are to use the same cutoff values for MSI and TMB across the world. We found it is particularly important to define minimum acceptance criteria for DNA quality and quantity when evaluating TMB.

How does a large panel such as the TSO500 affect laboratory efficiency?

AB: It's highly automatable, which means it can be built into a workflow that is mostly hands-off and left unattended to run overnight. The level of automatization also makes it extremely reproducible and allows for consistent results, and we have found it allows a 50 percent reduction in hands on time and a subsequent increase in efficiency.

LK: The complete workflow – from DNA isolation to final clinical report – takes us approximately six days. Although more expensive than small NGS panels, the larger panel provides results for many biomarkers at once. There is no need for sequential testing or multiple parallel tests, thereby decreasing the total turnaround time.

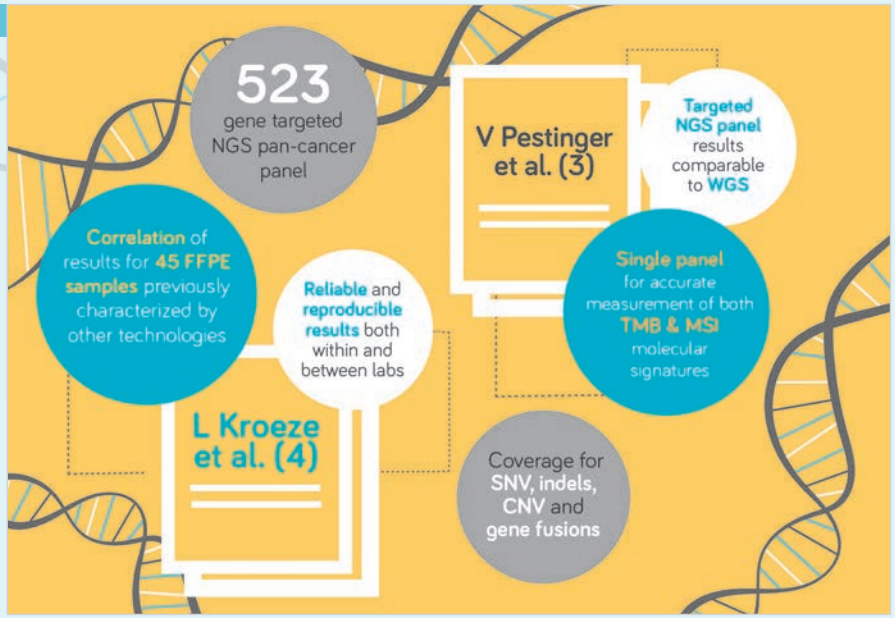
How did the TSO500 perform when analyzing multiple biomarkers and variant types simultaneously?

AB: Using a "TMB-high" threshold of 10 mut/Mb, the TSO500 classified samples with 100 percent accuracy. The panel was reproducible across multiple samples and tumor types and shows that a panel of this type would be suitable for the clinical determination of TMB status across different sample types and DNA inputs. The same can be said for MSI, which we detected in all samples that had over 10 percent unstable MSI sites.

The targeted RNA-seq assay component of TSO500 offers a unique advantage to detect known and unknown fusions events – and we reliably detected *NTRK*, *ALK*, and *RET* fusions. We think the hybrid-capture enrichment used in TruSight technology is superior to conventional pathology techniques for detecting fusions because you don't need to know the other end of the fusion breakpoint. As long as one of the partners is on the fusion panel, you can

work out novel fusions and find potentially pathogenic fusions that couldn't otherwise be detected.

LK: We compared the TSO500 results with our current NGS approach and were able to detect all previously determined mutations, amplifications, and MSI present in the samples. One of the main benefits of a larger panel is that less material is needed overall than for separate assays. For example, a lung cancer brush biopsy produces only a small amount of material – but the TSO500 maximizes the information obtained from that limited sample.



What advice would you give to anyone implementing the TSO500 into their workflow?

AB: I think a basic knowledge of molecular biology is helpful. You also need to have the correct equipment, which requires a small initial capital investment. In terms of workflow, the most important aspect is to work out how many samples you're going to process each week; it's not worth stepping up to an automated workflow if you're only doing a handful. If you process hundreds each week, then an automated workflow is the favored option.

LK: It's possible to manually analyze the list of variants produced by the TSO500 – but we built an additional bioinformatic workflow that annotates the variants and makes filtering easier. For that reason, the assistance of a bioinformatician was very helpful during implementation. I would also advise to optimize the DNA shearing which is especially important for reliable MSI calling, because the sequencing reads should be long enough to span the complete microsatellite regions.

What are the main advantages of performing CGP in-house?

AB: I think the primary benefits are speed and breadth of assay. Comprehensive panels would also support consideration of multiple novel therapy options. I would argue that, in many solid tumors, CGP will replace testing methods that use smaller gene panels. For example, colorectal cancer patients should be tested for *KRAS* and *BRAF* mutations – but limited panel sizes mean that doesn't always happen.

Although some pathologists question the standardization of assays that enable local CGP testing, we demonstrated that the TSO500 minimizes interlaboratory variability. Consistent results both within and between labs are obviously critical to devolve testing down to the local level. This kind of in-house testing provides quicker turnaround times, greater confidence in results, and easier communication with molecular pathologists.

LK: The main advantages of CGP are that less material is required, turnaround times are shorter without sequential testing, and there is a higher chance of finding actionable targets. We anticipate that this latter advantage will also result in more patients who are eligible for clinical trials, which ultimately leads to better knowledge of new therapies.

As molecular biologists, we prefer to analyze sequencing results ourselves so that we have a better feeling of the quality and reliability of results. This confidence is crucial when it comes to communicating with clinicians about the consequences of our molecular findings for therapy – and we can easily respond to additional questions that would ordinarily make life more difficult when liaising with an external organization. The highly reproducible TSO500 provides this reliability and unlocks the benefits of local CGP testing.

Andrew Beggs is a Professor of Cancer Genetics and Surgery in the Institute of Cancer and Genomic Sciences, University of Birmingham, UK.

Leonie Kroeze is a clinical scientist in molecular pathology in the laboratory of Marjolijn Ligtenberg at Radboud University Medical Center, Nijmegen, the Netherlands.

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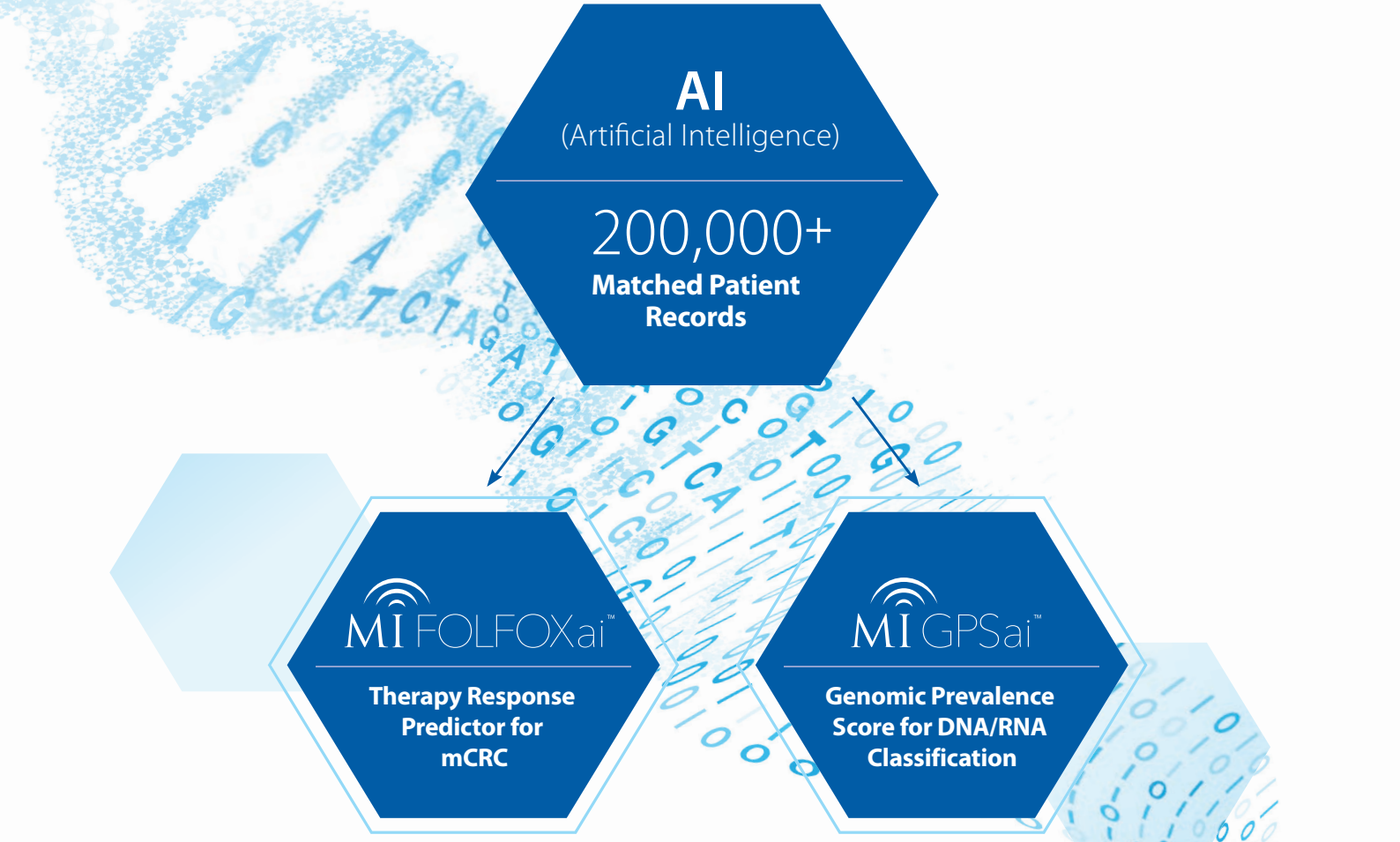

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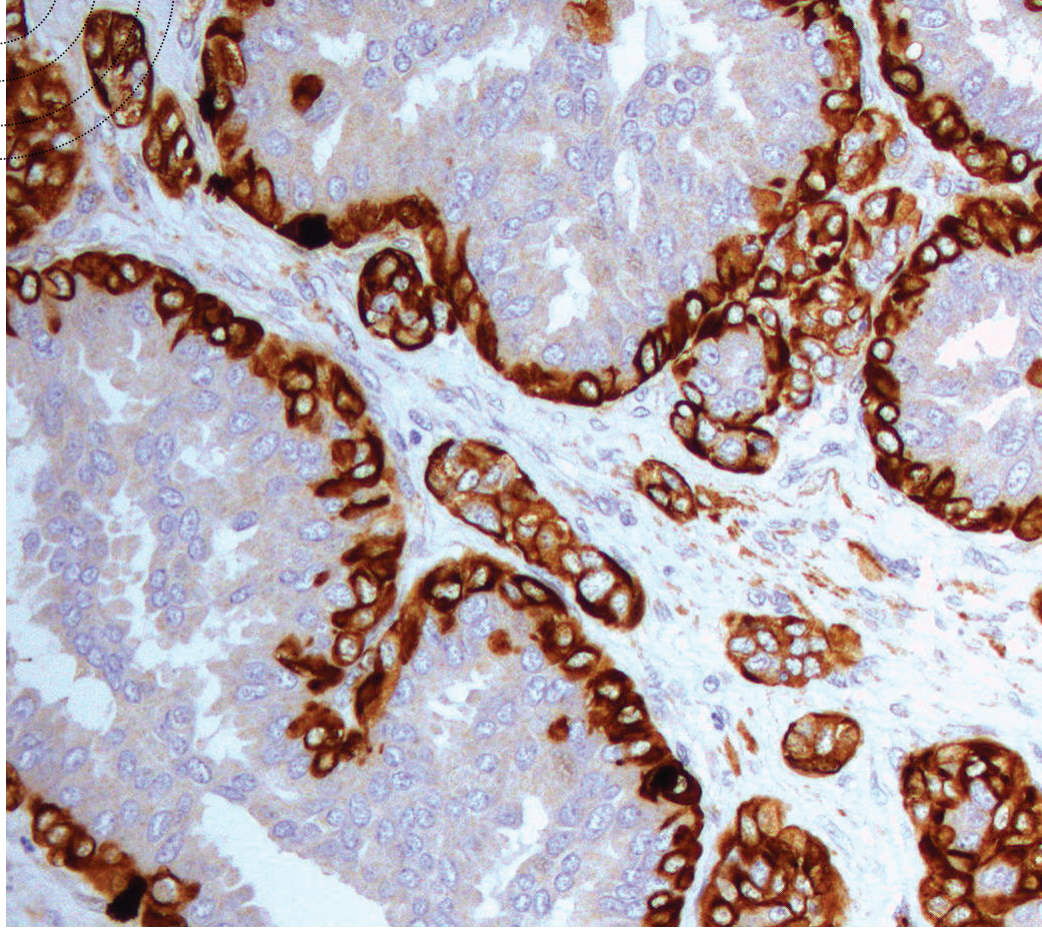
From Stain to Shining Stain

Four decades of progress in immunohistochemistry

By Cynthia Cohen



Credit: Wikimedia Commons user Nakos histopathology

For nearly 80 years, immunohistochemistry (IHC) has been a valuable tool for pathologists and laboratory medicine professionals the world over. In every stage of its evolution, we owe it many diagnostic debts – but I'm not here to document the complete history of IHC. For that, there are several excellent papers (1,2,3) to which I direct curious healthcare professionals. Instead, I would like to present a more personal experience: a journey I've shared with histotechnologists that traverses nearly five decades of IHC advances – from the early 1970s to the present day. After almost half a century, it feels appropriate to document the progress we have made thus far and note where we still have room to improve.

The IHC technique was developed from immunofluorescence (4,5,6) to light microscopy with the use of peroxidase/antiperoxidase (7) – an approach sometimes informally known as the "hamburger" because of the way antigen and antibody are sandwiched. My colleagues and I started using this method in the 1970s with our own, often very basic, problems.

Preanalytic parameters

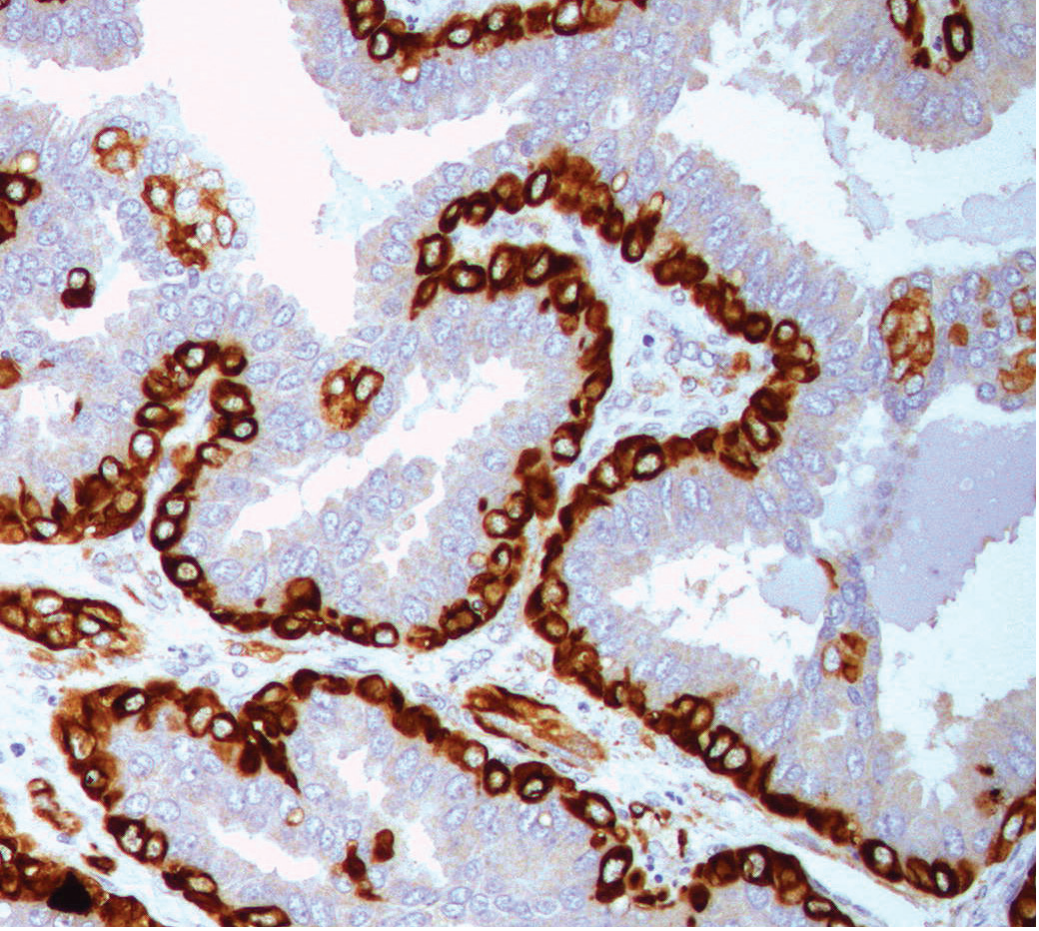
Adherence of tissue sections to slides

To get tissue sections to adhere to the slides during IHC, we tried several adhesives: white glue, egg white, and serum – each a messy and unreliable solution. Positively charged slides were a markedly better proposition, although we had to dip each one individually into polylysine or silanize it with a solution of 3-aminopropyl triethoxysilane in the presence of catalytic traces of water (8). Later, positively charged slides became available commercially; now, they are cheap enough to be used routinely on all slides cut in the histology laboratory – and so we're able to perform IHC over a hematoxylin and eosin (H&E)-stained or previously IHC-negative slide without de-staining or losing the section.

Optimization and validation

Originally, antibody optimization required the use of one or two positive control tissues in a checkerboard pattern with the other reagents just to ensure that the antibody worked. Nowadays, the process is more involved; we also perform validations (on 10 positive and 10 negative tissues wherever possible) to determine sensitivity and specificity. We have made a tissue microarray (TMA) of many different neoplasms and normal tissues that serves as our negative control and also shows tissues that are unexpectedly positive with the antibody.

There are now guidelines for the validation of all antibodies (non-predictive markers) prior to use on patient specimens (9), with at least 90 percent overall concordance required between the new diagnostic test and the comparator test or expected results. Users must test a minimum of 10 positive and 10 negative cases (10). For predictive markers such as estrogen and progesterone receptors (ER and PR) and HER2, even more validation is required – users should include 20 positive and 20 negative cases with ±95 percent concordance (11,12). Other quality parameters include proficiency testing, which we obtain from the College of American Pathologists for ER, PR, HER2, c-KIT, microsatellite instability (MSI), and hematopoietic markers. Online databases compare antibodies from different vendors, published literature indicates antibody sensitivity and specificity (although many papers omit what positive control is used), and accreditation processes all help acquire and maintain quality IHC.



Conferences and publications

IHC was, and still is, incorporated into general and subspecialty meetings. I was there when the United States and Canadian Academy of Pathology conference first became a "brown" meeting because of the many posters that used diaminobenzidine (DAB) as a chromogen in IHC. Today, the move is more toward in situ hybridization (ISH) and molecular studies (13,14). As a result, events specifically focused on IHC are finding a place. Since 2007, Hadi Yaziji and Richard Eisen have organized an excellent annual retreat exclusively for technologists and pathologists using applied IHC and molecular pathology (15), and a meeting of the International Society of Immunohistochemistry and Molecular Morphology, is in the pipeline.

IHC has also long been included in both general and subspecialty journals. Publications specifically designed for IHC include Applied Immunohistochemistry and Molecular Morphology, available online and as a hardcopy, and the forthcoming ISIMM Journal. For those who prefer to learn online, the Pathology Outlines website (pathologyoutlines.com) gives the expected immunoprofile for each neoplasm its authors discuss. Immunoquery (immunoquery.com) gives different antibodies and combinations with expected sensitivity and pertinent references for various neoplasms. ExpertPath (expertpath.com) is an online alternative to maintaining multiple sets of hard copy reference materials.

Analytic parameters

Antigen (epitope) retrieval

Antigen retrieval to overcome the effects of formalin fixation has undergone an impressive evolution. Initially, we used a five to 10-minute trypsin incubation; later, we moved to heating via a hot plate, which improved IHC, but resulted in background stain. We improved our results further by switching from the hot plate to 20–30 minutes in a microwave, steamer, or pressure cooker for most IHC, or 30–40 minutes in a hot water bath for HER2 (16). Much like early polymerase chain reactions (PCR), we began by performing all of these heating steps manually – but modern technology has given us the automated stainer, which controls time, temperature, and pH.

Antibodies

Antibodies were difficult to obtain in the early days of IHC. The best were those homemade by basic researchers, which we could dilute to 1:10,000 or more. We did try antibodies used in radiology and bought several from commercial companies – but they were like water; they never worked. A commercial alpha-fetoprotein antibody, given to me in 1972 in Copenhagen, did not work on several fetal livers of different months' gestation, despite what was written in the literature. The magic bullet was the alpha-1-antitrypsin antibody (AAT), which stained a liver from an AAT-deficient patient and set us on the path to success.

Since then, many antibodies have become available from many different and often very good companies. We even have ready-to-use antibodies for those who prefer them to concentrated antibodies requiring titration – and some are formulated especially for automated stainers. These make life easier for histotechnologists, who can now work more efficiently and with greater ease.

We have also improved antibody quality by refining our sources. From polyclonal rabbit anti-human antibodies, which resulted in a certain amount of nonspecific background, progress led us to mouse and then rabbit monoclonal antibodies, which are more sensitive and have less background (17).

IHC methods

We began with the direct immunolabeling technique, in which the labeled primary

The late David Brigati with the Codon – the first automated slide immunostainer. *Credit: ER Unger.*

antibody detects tissue antigen with a one-step protocol (not as sensitive as modern protocols and with nonspecific background). The indirect method of immunolabeling followed; in this approach, the bivalent primary anti-human antibody binds to cell or tissue antigens and to the labeled secondary antibody, which reacts with IgG from the species of the primary antibody. The indirect immunoenzyme peroxidase technique is similar – but the secondary antibody has a peroxidase label attached that causes DAB substrate to precipitate over hydrogen peroxide when both are introduced. The immunoenzyme-bridge technique has a third antibody made in the same species as the first and directed against peroxidase; this method uses the sandwich approach, in which peroxidase is added, followed by the peroxidase substrate. And, finally, in the peroxidase-antiperoxidase complex technique, the third antibody to peroxidase is added in a complex with peroxidase (7), which leads to increased sensitivity and decreased background. Ultimately, a long dextran polymer labeled with secondary antibody and multiple enzyme molecules can further increase sensitivity and avoid endogenous background biotin.

These techniques use similar enzymes: horseradish peroxidase, which is highly sensitive and gives a precise chromogenic reaction, and alkaline phosphatase, which is useful for tissues with pigment (as in dermatopathology) and for double staining. Originally, the chromogen of choice was the brown DAB, which is alcohol resistant. It was eventually suspected to be carcinogenic and its removal from the market was planned; although this never happened, we nevertheless changed to amino-ethyl-carbazole – but this red stain is not alcohol-resistant, resulting in a loss of stain when we used Permount rather than Aquamount for coverslipping. Today, we use Fast Red TR, which is alcohol-resistant, for dermatopathology specimens.

Automation

Our IHC run was initially performed manually in one or two 10x13" cake tins bought for less than US$10 each at a department store. We placed a damp hand towel under the metal slide tray. This assembly could be incubated at room temperature for 30–60 minutes or overnight at -4°C. We sometimes used a slide agitator,

which we hoped would result in evenly distributed stain on the slides (it did not). Once we needed to stain more than 40 slides (in two cake tins), we moved to automation. The technologist could not put one reagent on more than 40 slides and then return to add the next reagent without rushing or letting slides dry out, and mistakes inevitably occurred.

Elizabeth Unger worked with me in the anatomic pathology department at Emory University. She and the late David Brigati developed the first automated slide immunostainer, the Codon (18,19), which used capillary action. We then acquired a 250-slide stainer, which we nicknamed "Hartsfield Airport" for its size. Unfortunately, we found that it took hours to complete a run, so we only stained 100 slides at a time – spurring us to purchase a smaller 100-slide stainer for more practical use.

Around this time, we stopped being able to use microwave antigen retrieval, our standard technique prior to a run. Why? Because it was a patented technique... (Who allows people to patent a scientific method?!) As a result, we used first the steamer and later the pressure cooker. Fortunately, all of these heat antigen retrieval methods produced similar results. We performed our ER and PR IHC on formalin-fixed sections using the Codon (20,21). Results were not excellent, but they were interpretable (especially if positive), and no fresh frozen tissue was available. We also immunostained immunoglobulins and complement in fixed, paraffin-embedded sections of kidney biopsies. Results were good, but the technologist never instituted the new technique in the fluorescence laboratory, so we never got to see its performance on a larger scale.

Eventually, we got a 48-slide Dako autostainer that proved to be excellent. We currently have three and love them, although we do need to manually retrieve antigens beforehand. In addition, we use four Leica 30-slide stainers on which we can also perform antigen retrieval according to the necessary parameters – at low or high pH for 10 or 20 minutes. We use concentrated antibodies that we optimize ourselves wherever possible, but many excellent ready-to-use antibodies are also available. Finally, we have also used a Ventana Benchmark autostainer, which yielded excellent results for a research project on *BRAF* in melanomas (22). Each automated system has its pros and cons (23,24) – some are closed systems that incorporate antigen retrieval, but require company service for problems rather than being reparable in-house. Each laboratory must assess and choose according to its individual needs and budget.

Controls

For many years, each antibody required positive, negative, and internal controls (25,26). We have used tissues remaining from clinical surgical specimens, prior to incineration, for positive controls; we get blocks as soon as possible so as not to over-fix in formalin. If patient consent is required, as is now being suggested, there may be a problem for old blocks we still use, which only have a tissue and/or tumor type and sometimes a surgical pathology number indicated. Patient names are never used. Currently, for clinical research projects, patient consent is obtained by clinicians before any surgical procedure – which will make life easier for my colleagues down the line. We do occasionally get blocks of tonsil (from a pediatric laboratory) for the many hematopoeitic markers, syphilitic lesions (for *Treponema pallidum*), placenta (beta-hCG), and Kaposi's sarcoma (HHV8) to name a few, and we give blocks to other IHC laboratories as well.

Today, we are testing synthetic tissues for use as positive controls (27). The synthetic tissues are in a small TMA from two sites and two tumors; each core stains well for numerous antigens and reproducibility over five runs, when quantitated by image analysis, is excellent. We are now testing a TMA containing synthetic HER2 controls with three different levels of HER2.

We have used small TMAs that we have made from tissue cores of breast or endometrial carcinoma with negative, low, intermediate, and high ER, PR, and HER2 as positive controls. We use TMAs of tonsillar tissue from many different tonsils for optimizing hematopoietic markers and for validation. Other TMAs serve as negative controls and for validation. We do not include positive controls on the same slide as patient tissue; instead, we use one separate control for several cases (unless they come from a separate facility, in which case a positive control is sent with patient slides). We have attempted to cut positive controls at one end of the slide, but so far have not built up an inventory (28).

I remember, for many clinical research studies, having to buy antigen and then assess how much to use to adsorb the antibody, so that we had an adequate negative control to satisfy journal reviewers. This was assumed to be better than the serum or buffer we would otherwise have used as a negative control. Today, in the age of cost containment and standardization, we do not use negative controls with patient specimens, although they are used for optimization and research projects. This makes for a shorter run or more patient slides stained per run and reduces cost. When slides are stained in a panel, each can act as a negative control for the others. However, I clearly remember staining kidney frozen sections with the peroxidase/antiperoxidase technique and being delighted with the excellent results – only to find that our negative controls stained just as beautifully.

Antibody cocktails

We also perform IHC using cocktails (29,30,31,32), which is of immeasurable use in defining difficult-to-diagnose cancers. An example is PIN4, a cocktail of antibodies against high-molecular-weight keratin, p63, and p504S that we use to diagnose prostate cancer in needle biopsies. Cytoplasmic keratin and nuclear p63 are negative because basal cells are absent, whereas p504S (AMACR, racemase) stains the luminal cells. Although we have used commercial cocktails for this purpose, we now make our own with excellent results. We also use homemade cocktails of TTF1 and napsin A for lung adenocarcinoma; cytoplasmic high-molecular-weight keratin and nuclear p40 or p63 for squamous cell carcinoma; tumor pan-cytokeratin and CD31 for angiolymphatic invasion; and HMB45, melan A, and tyrosinase for malignant melanoma. By combining any two of our working antibodies, we can create a good cocktail for a particular use (30,33).

Cytology cell blocks

We perform IHC on cell blocks of fine needle aspirations (FNAs) and fluids, as well as surgical specimens, such as resections and biopsies (34). Results are similar and excellent – even after the use of a rapid processor for biopsies. We've only encountered problems with certain alcohol-based fixatives and transport media, but using formalin has improved the situation.

In situ hybridization

We can now do ISH on our automated stainers for kappa and lambda, human papillomaviruses 6, 8, 16, and 18, cytomegalovirus, and Epstein-Barr virus (35). For kappa and lambda, ISH is much more sensitive than IHC because of the absence of background; for human papillomaviruses it is also much more sensitive – but only as individual viruses, not cocktails; cytomegalovirus, in contrast, is better by IHC. Other organisms that stain well by IHC include adenoviruses, *Toxoplasma, Helicobacter, Treponema pallidum* (no more time-consuming searches), herpesviruses 1 and 2, and polyomavirus. Other viruses, such as high-risk papillomavirus, also stain well with RNA ISH using RNAscope and probe.

Carcinoma of unknown origin

We can now use IHC to identify site of origin of carcinomas of unknown primary (36,37), which allows us to help clinicians determine treatment approaches – for instance, if biomarkers of a particular cancer arise, if predictive markers are present, or as a companion diagnostic to suggest that a patient may respond to a particular treatment (for instance, anti-PD-L1 therapy) (38).

Molecular markers

IHC can be used as a surrogate for molecular testing. Molecular markers we have studied by IHC include MSI (39), wherein five Barrett's esophagus-associated adenocarcinomas showed loss of *MLH1* and *PMS2* expression; all of those showed high-level MSI by PCR and four showed *hMLH1* promoter methylation. IHC was 92 percent sensitive and 89 percent specific compared with *MYC* rearrangement by FISH in 31 lymphomas (40), and *ALK* was 100 percent sensitive and 96 percent specific in 110 non-small cell lung carcinomas (41). Molecular IHC surrogates are also useful in breast cancer (42,43).

Post-analytic parameters

Image cytometry

IHC quantitation can be performed by visual assessment of intensity, percent positive, and distribution or by image cytometry, which eliminates the human element. Over the years, we have used the CAS 200 (Dako), ACIS Chromovision (Dako), and now Aperio (Leica) to quantitate breast markers, but we still review each slide visually as well to ensure that the correct areas are quantitated and results are similar by both methods. Other situations where image cytometric quantitation with digitization are used include *MIB1* proliferation in breast carcinoma, brain, and neuroendocrine tumors – although some feel the latter is best quantitated manually (44). Thus, the qualitative method has become quantitative, requiring even more standardization and validation.

Whole-slide scanning and digital imaging

We already use digital imaging for a plethora of reasons: photography of gross specimens, reports, microscopic pictures used in teaching, tumor registry meetings, sharing of cases, and on a daily basis for quantitating breast cancer IHC markers and ordering IHC with generation of a daily list. Sections are still cut, stained/immunostained, and delivered to residents and pathologists, but the workflow is now documented by scanning barcoded slides on their journey from gross room to laboratory to microscope. We do our immunostaining on automated machines, each method documented and controlled by computer (46). Thus, we are moving closer to the Lean laboratory of efficiency, quality, and high productivity. We performed image analysis for ER quantitation 25 years ago (45), but we never dreamed we would be running like an automated Toyota Corporation (47) today – the lab of the future!

In the early days, we had to ask a photographer to take pictures of microscopic slides and wait at least two days for the resulting kodachromes

(48). Later, we purchased a Lasergraphics film scanner and could deliver the whole film, taken with a microscope camera and processed by the Lasergraphics machine, to the camera store. That took only a single day to develop. Now, the digitized microscopic image is photographed and available immediately online for a poster, talk, registry meeting, or as a print for a manuscript. It used to take three months for the residents to assemble their pathobiology talks; now, they are done in days. Speaking of talks, we used to have to ask the photography department to make diazo kodachromes (49) of our typed descriptions, all of which went into a carousel and often stuck during presentations. No such problems now… as long as the computer functions well!

Whole-slide imaging received FDA approval in April 2017 for primary diagnosis (50) and it has taken off ever since. I recently saw a webinar where the imaged IHC slide was viewed on the monitor together with the H&E images for easier diagnosis!

Companion diagnostics

Increasingly, companion diagnostics are becoming a valuable application of IHC (51). Pembrolizumab was initially FDA-approved for use with metastatic – and now also primary – non-small cell lung carcinoma (NSCLC); PD-L1 as a companion antibody shows strong intensity and >50 percent of cells are immunopositive (52). Pembrolizumab is also FDA-approved as a complementary diagnostic in metastatic melanoma and PD-L1 and nivalumab in NSCLC, metastatic melanoma, Hodgkin's lymphoma, and urothelial, renal cell, and head and neck carcinoma independent of tissue PD-L1 expression (52,53). Two FDA-approved PD-L1 antibodies are now available from Dako (clones 22C3 and 28-8), but at high cost of $3,500–4,000 per 50 tests. Other antibodies from different companies, with different cutoff values and for use with different carcinomas and therapeutic inhibitors, are also available (54,55).

Techniques

Techniques that may not yet be fully integrated into clinical anatomic pathology include miRNA, RNAscope for RNA ISH (with very expensive probes, but a large selection), and molecular methods (56), many of which can be done quicker and cheaper by IHC, MSI, and digital imaging with whole-slide scanning.

We have done technical validation studies using RNA ISH for 18 high-risk papillomaviruses. 100 percent of p16 IHC-positive and 50 percent of p16 negative head and neck squamous cell carcinomas were RNA ISH-positive with 88.9 percent concordance with p16 IHC (13). We obtained excellent, easy-to-read results (see Figure 3). Our EBER and CMV ISH studies showed 90.3 and 66.7 percent concordance with their IHC equivalents, respectively. Eight of 16 (50 percent) negative CMV IHC were positive by RNA ISH (57).

It seems that, for viruses, RNA ISH is a valid alternative to IHC – but concordance for cancers is much lower, particularly in the case of PD-L1 testing. Whereas 90 percent of lung adenocarcinomas and no metastatic colon carcinomas were PD-L1-positive by IHC, 60 percent of the same lung cancers and 25 percent of colon cancers were PD-L1-positive by RNA ISH (58). For other applications, RNA testing proved more reliable; TTF1 and napsin A IHC and RNA ISH were both 95 percent sensitive and 100 percent specific (37). Albumin RNAscope was 100 percent sensitive and specific for hepatocellular carcinoma (59), but TFE3 RNAscope was not a viable alternative to IHC or FISH (60).

The capture of circulating tumor cells and their genetic material has shown progress with improved technical and sequencing-based methods, bringing the possibility of liquid biopsy of solid tumors closer to reality. However, its clinical utility for diagnosis and treatment is still unproven (61).

Detection of protein in formalin-fixed, paraffin-embedded (FFPE) tissue by IHC is semi-quantitative. The possible future use of protein biomarkers detected by proteomics combined with IHC from FFPE tissues is exciting. A novel amplification system enables quantification of protein by counting dots (62) and can be combined with IHC. Combining IHC with mass spectrometry in the same tissue section allows highly multiplexed IHC (using three or more monoclonal antibodies) for direct quantitative imaging (63). For HER2, results from this approach were comparable with or better than the reference methods of ELISA and flow cytometry.

I can only imagine, with further technological advances, what the next decade – let alone four – will bring to the diagnostic laboratory. We are already in line for diagnostic whole-slide imaging, with FDA approval in the United States and several laboratories in Europe already using it. This digital approach will include scanning of all IHC-stained slides – unless, that is, we go entirely to molecular testing. Personally, I don't see that as a possibility; I believe that anatomic pathologists will still want to see the tissue in H&E and immunostained slides, whether on a computer monitor or through a microscope.

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Please see references online at: tp.txp.to/stain-stain

Precision Oncology Genomic Profiling: In-House or Centralized?

Panel discussion highlights the benefits of keeping it close

Targeted and immuno-oncology therapies requiring biomarker testing have proliferated in the last decade. Healthcare providers now face a complex decision: whether to outsource this new testing to centralized laboratories or implement it in their own labs. What is best for the system – and what is best for patients?

To explore these questions, we invited expert pathologists Fernando López-Ríos from Spain, Ruthy Shaco-Levy from Israel, Michael Vieth from Germany, and clinical scientist Philip Bennett from the UK to a panel discussion to share their views and experiences. They've participated in broadly different approaches to the issue, from limited testing hubs within a single country to plans to completely outsource testing to overseas commercial labs.

The discussion was moderated by Michael Schubert, editor of The Pathologist, and Luca Quagliata, head of medical affairs for Thermo Fisher Scientific's clinical sequencing division.

What does in-house testing mean to you when it comes to oncology?

RS-L: Performing all the pathology assays in my lab, from the hematoxylin and eosin (H&E) stain to the immunohistochemistry and molecular tests, is what appeals to me about in-house testing. It means correlating the molecular analysis with other clinicopathologic features to see the whole picture. For example, in breast cancer, we report on tumor size, tumor grade, and receptor status. For consistency and efficiency, molecular pathology should be included in that report – and performed on site.

FL-R: In-house testing means controlling the whole testing workflow so that you can influence turnaround times and other critical factors. In my opinion, it also enables us to put patients a the heart of the care process.

What is your institution's approach to precision oncology testing?

RS-L: In my department, we perform all our molecular assays locally, because it benefits everyone involved. Turnaround times are shorter, there's no need to send out precious samples, and clinicians can directly discuss test results with pathologists. The pathologists get to work with advanced technologies and fully develop their professional skills. All parties appreciate the high-level pathology reports with clinicopathologic correlation.

MV: Our system is driven by clinical and patient needs and follows a basic rule: all tests that can be performed locally should be. If we encounter any problems with testing, a nearby university hospital can help us, but we try to carry out all routine tests in-house so that we build the expertise to handle not only simple, but also more complex cases.

What are the pros and cons of a centralized test model versus locally conducted testing?

PB: Centralized testing makes sense, for example, with a homogenous liquid biopsy or for certain biomarkers that are too rare to implement cost-effectively in every local laboratory. Unfortunately, some samples are sent out to hub laboratories – who potentially have lengthy turnaround times and could lack to the pre-analytical assessment capabilities that some cases need – just to get the basic standard-of-care biomarkers. This is a waste of resources. We must focus on doing those routine tests quickly, cost-effectively, and as locally as possible.

FL-R: With the advent of NGS panels, genomic profiling has become more leaner, cheaper, and more user-friendly. Everything is quicker in-house, with much less chance of losing important material or information. One of the best arguments for in-house genomic profiling is the control it affords over the preanalytical parameters, tissue specimen selection, and sample quantity.

MV: I also see an ethical issue with sending samples to commercial laboratories abroad. In Germany, the healthcare system is over 90 percent publicly financed and, if you spend this money outside the system in which it was generated, you aren't supporting it and enabling its development – and this is an ethical problem.

Do you see value in increasing local knowledge and expertise in molecular testing?

RS-L: Yes. Pathology is one of the fastest-developing fields in medicine and molecular pathology is one of the fastest-developing areas in pathology. Soon, molecular pathology will likely be routine for confirming the diagnosis and prognosis of most tumors. Pathology departments not using these techniques will be left behind, so pathologists must develop expertise with the new testing methods – and with molecular pathology in general.

Have you experienced a move toward test centralization in your country?

RS-L: My hospital is part of a chain of

institutions, and a few years ago, the decision was made to centralize our molecular pathology. The new central laboratory was not equipped to handle our testing needs, nor was it connected to our pathology department. Clinicians were not happy with the results or the fact that they could not properly discuss tests with the pathologists who had performed them. Eventually, the complaints mounted and the centralization attempt failed.

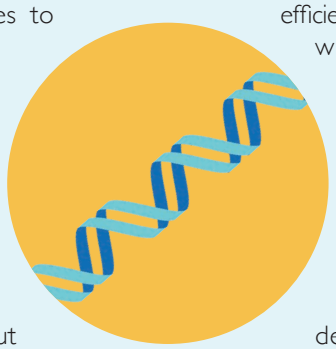
While this effort was underway, a wide gap developed between my hospital chain's capabilities and those of hospitals whose labs had not been centralized and it took us some time to catch up.

How can in-house testing benefit your interaction with your colleagues – for example, in multidisciplinary teams coordinating oncology patient care?

FL-R: When we started NGS, we set up an internal "intra-laboratory molecular tumor board" to discuss test results before reports are released and clinicians and patients apprised of the results. It's a formal meeting among the molecular biologists, pathologists, and technicians; we integrate the pathology information and individual biomarker testing with the NGS results and make sense out of the huge amount of information. This facilitates efficient conversation with not only our clinical colleagues, but also our patients, enabling them to understand and get the best value from their test results. It worries me that some people treat NGS results like something simple and straightforward, and think it's enough to just send results to clinicians. These are complex tests with a lot of information that must be interpreted and put into context for every patient's clinical situation and pathology context.

Reducing turnaround time (TAT) to result is a hallmark of in-house testing. How important is it?

PB: No clinician ever complained about high-quality results arriving too quickly for any test, oncological or otherwise. It clearly impacts patient care. But it is also important to understand that the speed at which your lab can operate is not the only factor influencing TAT. Under General Data Protection Regulations, if you deal with cross-institutional or cross-IT systems, you are likely to encounter test result delays. The same applies to transporting samples.



What's the value of keeping samples at your institution?

PB: This is the ideal scenario, and one of the problems with planned centralization is that people do not want to send out tissue blocks. However, if you outsource sections, curls, or slides from those blocks, you may be wasting material and not meeting preanalytical or sample requirement needs. Kept in-house, we can ensure that testers take only what they need from each sample.

RS-L: It is important to preserve as much as possible of precious patient samples. If you do your testing in-house, you can decide on the test flexibly based on amount of sample available. The centralized labs perform the same large over 500 gene panels on all samples and sometimes do not get any result as there just was not enough of the tumor material This means possibly re-biopsy for patients and further delays.

Can any pathology laboratory today do genomic profiling for key predictive markers?

PB: From a technological point of view, I think we are near. The latest developments in PCR and NGS equipment are very much "sample in, result out." However, it will differ by country depending on the healthcare model. In the UK, following the 100,000 Genomes Project, there is substantial movement toward a few centralized molecular pathology laboratories. Some laboratories like ours, with existing skills and high sample volumes, are trying hard to "stay in the game" – but existing public sector pathology laboratories without molecular capabilities would probably struggle to establish them now.

FL-R: I agree. From a technical perspective, I can imagine that molecular profiling by efficient, actionable NGS panels will be relatively easy for most laboratories within a few years. But it all depends on how health systems organize their workflows. Currently, in Spain, most institutions have their own budgets and make their own decisions, but it's a very mixed picture.

RS-L: In Israel, currently most, even small labs can do molecular tests with simpler methods such as PCR, FISH, or NGS assays. Larger academic hospitals perform NGS. I think in future, NGS will likely become even more routine and will be done even in smaller hospitals, because it makes sense for the clinicians and their patients as well as for pathology labs.

MV: With the recent advances in techniques and technologies, most pathology labs can certainly do NGS. It has to be cost-effective, of course, and you need qualified personnel – although not necessarily bioinformaticians these days.

Do you have a take-home message to share?

FL-R: I'd like to advocate for seeing things from the patient's perspective. When we offer patients an NGS test, we also offer to discuss it with them. That tends to reassure them, because they value our honesty about the pros and cons of different treatment options, expectations, and possible problems. Ultimately, we need a patient-centered system, and that can only be achieved if we keep molecular profiling in-house.

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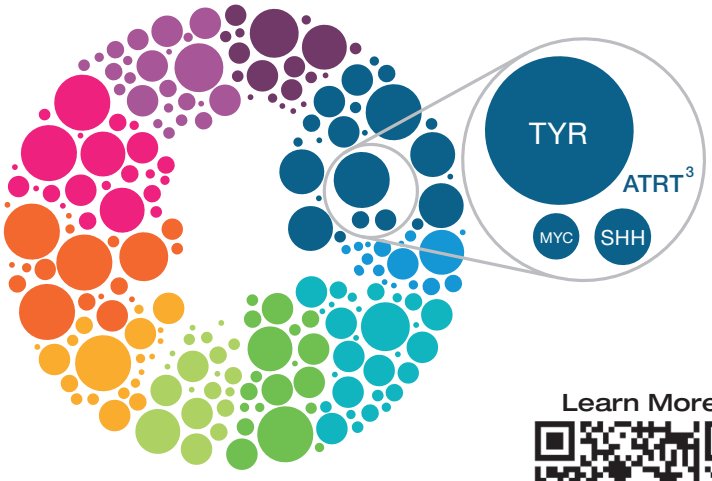
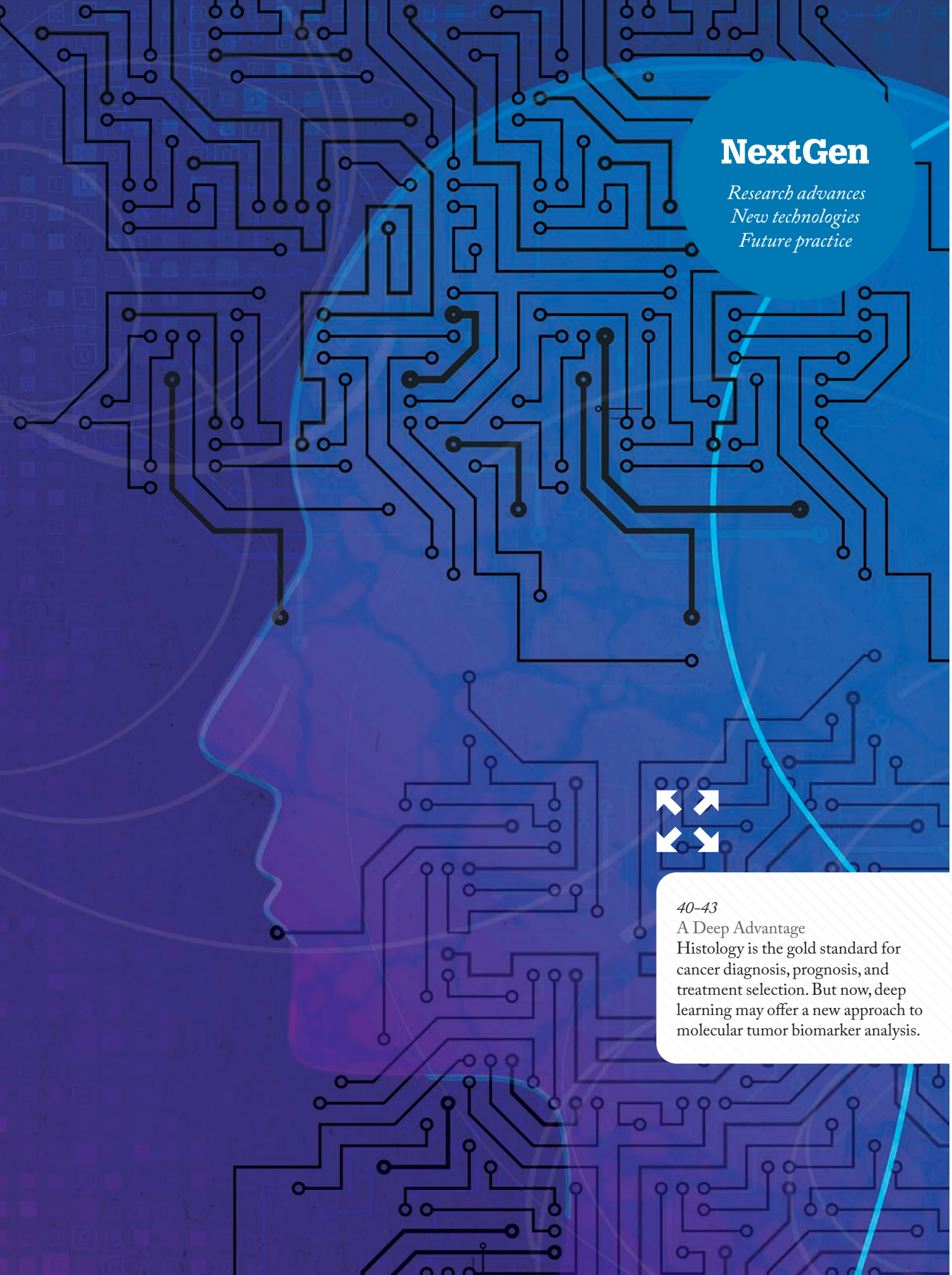


Learn More

Footnotes:

1. https://www.molecularneuropathology.org/mnp/classifier/1
2. CNS Tumor Categories map to WHO Guideline entities
3. Ho B, Johann PD, Grabovska Y, et al. Molecular subgrouping of atypical teratoid/rhabdoid tumors-a reinvestigation and current consensus. *Neuro Oncol.* 2020;22(5):613-624. doi:10.1093/neuonc/noz235



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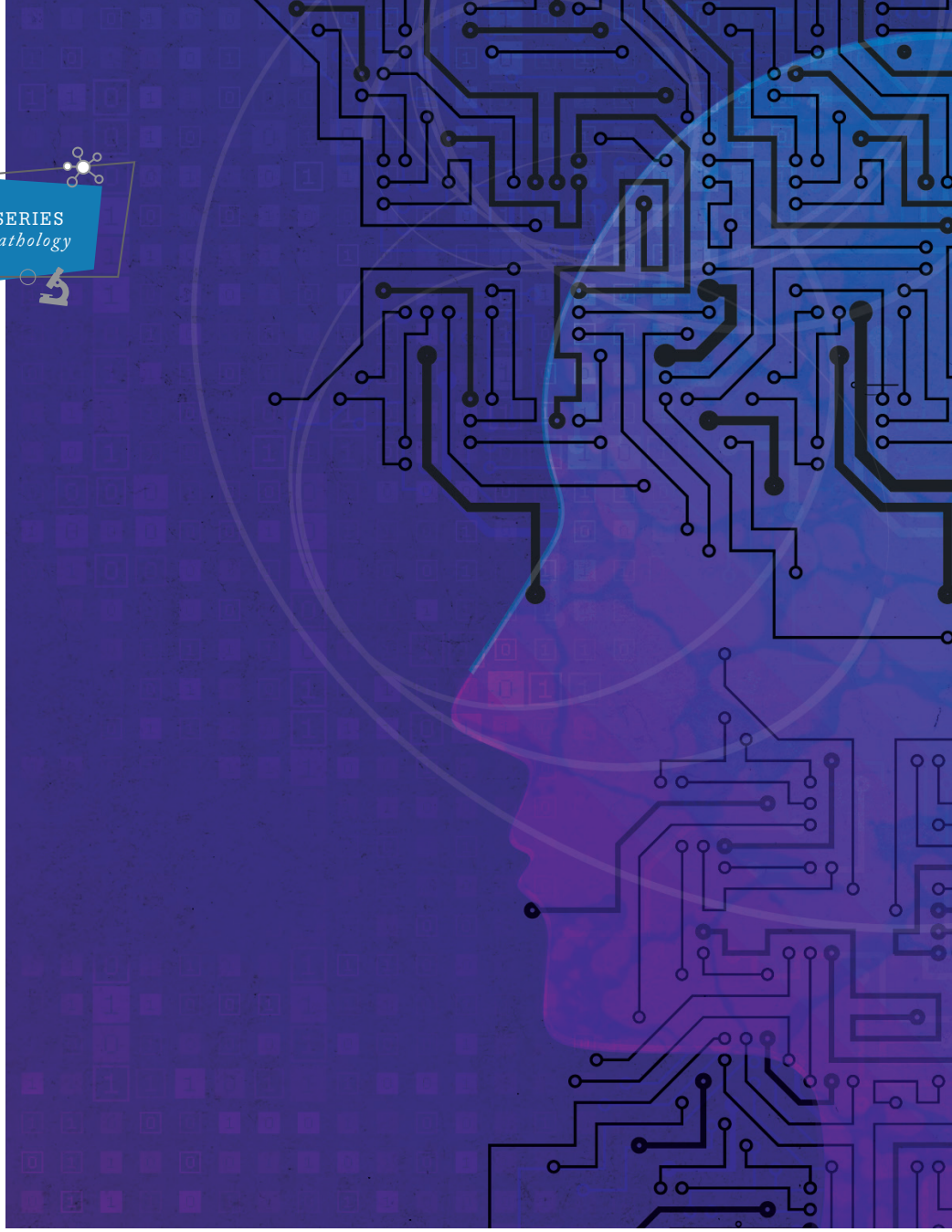
A Deep Advantage

How deep learning can help predict molecular tumor biomarkers from images

By Heather D. Couture



When choosing a treatment to target an individual tumor's specific weaknesses, its molecular properties are critical. And yet, despite the importance of this information, our most accurate methods of assessing molecular properties are expensive and often not routinely performed. We need a better solution – and artificial intelligence (AI) may offer one. Using AI to examine microscopic images of tumor tissue could provide a more cost-effective way of identifying individual tumors' molecular characteristics to inform treatment decisions.

Histopathology, of course, is the gold standard for diagnosing cancer. A pathologist, examining a prepared tumor sample slide under a microscope, decides based on appearance whether or not cancer cells are present. Although pathologists are experts in diagnosing cancer, they can still assess only limited tumor properties, even with the aid of microscopes and special stains. At the University of North Carolina, we investigated whether a computer could find features to predict molecular biomarkers – features too complex for pathologists to assess visually. Our answer, with a focus on breast cancer, was yes – and other researchers have recently found that the same is true for other cancer types.

Molecular subtypes for tumor stratification

One goal of cancer research is to identify cancer subtypes based on the characteristics of tumor cells. A better understanding of these subtypes can help identify both causes of and potential treatments for the disease. They also allow health care teams to personalize treatment to the needs of each patient by selecting the best approach for the disease subtype and prognosis.

Recently, we've seen great success stratifying tumors into subtypes using their molecular properties. For example, breast cancer is divided into five different subtypes that add prognostic information and treatment efficacy insights (1). Normal-like and luminal A and B cancers are hormone receptor-positive and generally have the best prognosis. HER2-enriched ones are often successfully treated with therapies aimed at the HER2 protein. Basal-like cancers tend to be more aggressive and have a poorer prognosis. However, the technologies we use to assess these molecular properties are expensive and the analyses are time-consuming. Most are not routinely performed – meaning

"To go beyond the complexity of what human experts can assess visually, we must rely on new image features."

that some patients who could benefit don't receive the testing – and labs with limited resources may not perform them at all. To complicate things further, tissue is often a limiting factor; in many cases, only a small amount of tissue can be excised from a tumor, leaving none for additional analyses beyond microscopic examination.

New studies keep identifying more molecular properties of potential clinical value, each requiring its own tissue sample and processing procedure – but current workflows are not designed to incorporate this many tests. Although comprehensive molecular testing will be difficult to implement at scale, tissue staining is common practice and imaging of such samples will become increasingly available as more laboratories transition to digital pathology.

Histological imaging for diagnosis

Hematoxylin and eosin (H&E) slides are inexpensive to produce and have a short turnaround time, which is why it is so well-established

and widely used to diagnose cancer, assess its aggressiveness, and study tumor tissue. However, pathologists cannot use H&E staining to assess the molecular biomarkers that guide treatment. Image analysis methods can provide a screening mechanism to identify patients who would benefit from further molecular testing or act as an alternative when molecular analysis is not possible.

Right now, we can use H&E to look at the size and shape of cell nuclei, their arrangement, the presence of other tissue structures (such as glands), and the presence of specific cells and cell structures (such as lymphocytes or mitoses). But, to go beyond the complexity of what human experts can assess visually, we must rely on new image features – and we must learn those features from the images themselves. This is known as feature or representation learning. The best-known (and currently most powerful) tool in this set is deep learning.

The rise of deep learning in histopathology

A computer can learn patterns in images so that it can make predictions based on those patterns – this is the essence of deep learning. After training on a data set of images and labels (such as biomarker status), the model can predict these labels on new, never-before-seen data. The model consists of multiple layers of features, with higher-level concepts built upon the lower-level ones. Going up the hierarchy, the features increase in both scale and complexity. Similar to human visual processing, the low levels detect small structures such as edges; intermediate layers capture increasingly complex properties like texture and shape; and the top layers of the network can represent objects and complex properties like tissue architecture.

This method has previously shown success for finding mitoses, segmenting tissue types, and detecting tissue structures. However, those features all have one thing in common: pathologists can see them under the microscope. The next step forward is to apply this powerful technique to higher-level (even tumor-level) properties for which a pathologist cannot provide detailed annotations.

Deep learning performs very well when given a lot of training data – often millions of examples. However, it struggles when (as is usually the case with medical images) training data is limited. A common solution for small datasets is "transfer learning": a model is trained on one data set, usually a large benchmark set of photographs called ImageNet, and then applied to a different one. In my work with breast cancer, I used the pre-trained model to compute features on H&E images and then trained a classifier to predict the biomarkers. Subsequently, I fine-tuned the model for improved performance on breast cancer.

Before working on breast cancer, my first experience with image analysis for pathology was in trying to predict melanoma mutations from H&E images. We used handcrafted features – the size, shape, and texture of nuclei and their spatial arrangement – properties that a pathologist looks for when diagnosing and grading cancer. Ultimately, however, we were not able to distinguish tissue samples with the mutations from those without.

This was in 2012, when deep learning was in its infancy. The research that popularized deep learning was published that same year (2), and open-source toolkits started to become available over the next few years. It was not until 2015 that today's more easily accessible libraries were first released. These developments in machine learning laid the groundwork for many pathology applications, including biomarker prediction from H&E.

Molecular biomarker prediction with deep learning

Recent work at New York University has shown that the exact mutations we looked at in melanoma are predictable from H&E images – using deep learning (3). Similar results have been found for point mutations in breast, colorectal, gastric, lung, and prostate cancer. A team at the Cleveland Clinic found that tumor mutational burden – a

"Recently, we've seen great success stratifying tumors into subtypes using their molecular properties."

measurement of mutations carried by tumor cells – is predictable for bladder cancer (4). Detecting mutations is typically done with DNA sequencing, which is costly and time-consuming, but can guide targeted therapies.

Our work at the University of North Carolina addressed genomic subtypes of breast cancer. Gastric, lung, and colorectal genomic subtypes have also seen success at other institutions. Genomic subtypes stratify patients based on the activity level of specific genes, providing prognostic information and helping to guide treatment decisions. Although assays are available for some genomic subtypes (5), RNA sequencing is still the most accurate method for distinguishing between the subtypes.

Going beyond mutations and genomic subtypes, we used deep learning technology to predict estrogen receptor status on breast cancer. Other researchers have since tested a simpler method on a larger data set and further expanded to include an additional 18 protein biomarkers (6). The standard method for assessing protein biomarkers is immunohistochemistry – an alternative tissue staining methodology that is time-consuming, costly, and requires a pathologist's subjective interpretation.

Even the presence of viruses can be detected from H&E images. Human papillomavirus was identified in head and neck cancer and Epstein-Barr virus in gastric cancer (7). Both viruses are major causes of human cancer and knowing about their presence impacts treatment decisions. Molecular tests for these viruses are expensive and not available everywhere – which leaves many laboratories reliant on staining to confirm their presence.

Deep learning creates new possibilities

The first step in each of these approaches is to identify the tumor regions in each tissue slide. Some groups rely on pathologists to delineate the tumor; others automate this step of distinguishing tumor from non-tumor tissue. All groups rely upon deep learning's abstract features to predict the molecular biomarkers. Traditional handcrafted features are just not powerful enough for these tasks. It is only through more complex properties – visible to machines, but beyond the capability of even the most expert pathologists – that we can now screen for some molecular biomarkers from H&E alone.

Deep learning is opening up a new world of possibilities in capturing properties that are too complex for human pathologists. It is a possible screening opportunity for any marker that can guide further testing and treatment selection. Although pathologists are – and will always remain – the experts in their craft, these additional insights can assist in targeting therapies for each patient.

Heather D. Couture is the founder of machine learning consulting firm Pixel Scientia Labs, which solves image analysis tasks for pathology applications. She recently completed a doctoral degree in Computer Science at the University of North Carolina at Chapel Hill, North Carolina, USA.

"Deep learning is opening up a new world of possibilities in capturing properties that are too complex for human pathologists."

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Digital pathology is not a magic wand; it must be carefully considered – just like any other process improvement

By Jane Rendall

With digital transformation at the top of the healthcare agenda, it can be easy to think that technology can solve the problems the laboratory faces. Just digitize and then everything becomes easier, faster, and better connected – right?

That's certainly a possibility. As managing director in an imaging company engaged in regional digital pathology programs around the world, I could describe plenty of real-world benefits digitization can deliver. From being a precursor to the application of artificial intelligence to supporting faster diagnoses and allowing instant access to opinions and reporting capacity from pathologists who might be many miles away, digital pathology has enormous potential. And, as pathology services remain under pressure to restructure, regionalize, and consolidate, digitization provides a means to help reshape structures in which services have been historically isolated in a single organization.

The challenge is that technology cannot do any of this successfully alone. Unless people with decision-making authority give rigorous thought to appropriate service design, workflow, clinical strategy, and the intricate requirements of different specialties, the chances of realizing digital pathology's service-transforming benefits are slim.

When digital could be damaging

A recent conversation I had with one cellular pathologist challenged my own thinking on the subject and reminded me about the dangers of viewing technology as a solution in itself.

Luisa Motta is a pathologist and strong advocate of the potential for digitization to really help a discipline faced with an insurmountable workload and massive resource pressures – especially as much of the existing workforce approaches retirement and recruitment of new clinical graduates proves challenging. Nonetheless, Motta is rightly concerned that digitization and service consolidation could be hugely damaging if not approached correctly.

The creation of regional pathology hubs and pooling of scarce resources has long been an ambition in healthcare worldwide. For example, Lord Carter made a strong case for consolidating pathology services to "improve quality, patient safety and efficiency" in his 2008 independent review of National Health Service (NHS) pathology services in England (1). As regional approaches now start to emerge, for Motta – and, I suspect, many other pathologists – the thought of being moved from the hospital to a business park off a motorway is unsettling. And it's not because they aren't willing to work as part of a regional network; it's not a protectionist approach to their own hospital's resources; and it's not because they are resistant to change. It's because they want to defend a vital mechanism of communication between the pathologist and the clinician – a relationship that is instrumental in decisions about patient care.

Don't sever the links

To alleviate such concerns, those leading transformation need a detailed understanding of how different pathology specialties operate and interact with clinicians as part of the clinical, surgical, or patient pathway. They need an accurate understanding of the profiles of different hospitals. They need to make sure they protect knowledge networks for which geographical proximity is important. And they need to ensure that relocation and consolidation of resources does not detract from service value, improvements, research, or the ability to discuss individual patients.

Consolidation and transformation must follow clinical strategy – and that may vary from one hospital or region to the next. Some regions, for example, may have hospitals with responsibility for specialist pathways and areas of highly complex surgery. For specialist pathologists like Motta, the idea of moving pertinent pathologists outside the hospital makes no sense, because having consultants close to the lab promotes discussion. In these complex areas, consultants actively visit the lab to see the surgical specimen – not just a slide or image – before it is dissected and discuss it with the pathology team. This act is difficult to replicate through a report on a digital image sent from a business park…

In more straightforward reporting cases that rely only on slides, relocation is less of an issue. But in specialist pathways and complex surgery, some pathologists are concerned that digital transformation could sever links and take them out of the clinical conversation. And, in some cases, if the clinical dialogue is damaged and the service becomes less effective as a result, pathologists worry that the service itself could be taken over by another provider. As Motta told me, "We cannot consolidate to the detriment of patients or the development of the service. Patients could be put at risk if we can't discuss things that are pertinent to the case."

Of course, most pathologists recognize the very restricted financial envelope of organizations like the UK's NHS, and the consequent need to save money. But the primary driver should not be financial gain. The bigger opportunity is to use digital pathology to achieve regionalization that allows quicker access to specialist opinions as part of the first report, rather than making consultants – and patients – wait for review after review after review.

For many pathologists, the main reason to consider transformation should be to eliminate duplication. In some systems, pathologists may refer reports from one

to the next, making patients wait weeks for a diagnosis. The real opportunity is to streamline pathways and develop a workflow that adopts the principle of getting it right the first time.

Don't apply a single strategy

How can we make this happen effectively? Success relies on having the right strategy to address the requirements of different services both locally and regionally, so that we don't end up in a situation where physical backlogs are converted to digital backlogs. Used properly, digital pathology enables the development of bespoke solutions to solve the clinical issues that a specific pathway in a specific region may be experiencing. If you understand the workflow and the pathway, and have gone through individual pathways to identify duplication, you can make informed decisions about where pathology labs need to be created, consolidated, or retained, and how they can be connected via digital pathology to ensure optimal use of resources – particularly workforce.

Motta cautions that some people focus too much on how many labs are needed and the cheapest way of creating them, rather than paying attention to workflow and pathways. Instead, she advises, ask what makes sense, what doesn't, and what the best system would look like.

She also believes that consolidation can be overdone to the point where benefits start to become risks. Sensible consolidation is determined by the development of bespoke solutions (centralist or federalist models) based on a deep understanding of the unique characteristics and requirements of patients and services in a specific geographic area – including how patient care is delivered and shared across organisations. The result should be streamlined patient pathways with minimal or no duplication, increased quality and sustainability, and avoidance of unwarranted variations in care. Centralist models that remove histopathology from tertiary hospital services and don't streamline pathways are problematic because they are not patient-centered and create inefficiencies in the larger ecosystem.

The impact of histopathology in patient care (even tertiary care) cannot be easily measured; value-based histopathology is a poorly developed topic with only rudimentary metrics and data. However, a working environment that facilitates informal interdisciplinary communication is good for patient care. Geographical proximity creates spaces for spontaneous communication. Meeting someone unexpectedly offers an opportunity to discuss interesting or challenging cases, quality improvement ideas, and more. These interactions are essential at a tertiary care level, where knowledge generation is an important responsibility in which histopathology plays a key role. Spontaneous interactions are also opportunities for ad hoc education of colleagues, including the new generation of health care professionals. Given the general lack of understanding of our role in patient care (which leads to misinformed policymaking, a lack of funding, and effects on service development and patient care), our visibility goes hand in hand with influence and survival.

There is an optimum consolidation point that allows you to work with partners to produce an initial report that is timely, accurate, complete, and allows the patient to move to the next part of the pathway. A deep understanding of the regional workflow – in particular, which specialties can be removed from the hospital and which cannot – is essential.

Do we have the right leaders?

Many of the people currently leading transformation understand its intricacies. As digital pathology begins to move beyond discussions on whether digital images are as effective as microscopes and slides and focuses on the reality of transforming services so they can cope with demand and better serve patients, informed leaders will be key to making such transformations work. But there can be no room for complacency; in large and complex institutions like the NHS, we must continually challenge leadership structures to ensure that they have the necessary knowledge, skills, and commitment.

This need has not gone unnoticed. Matt Hancock, the UK's health and social care secretary, alluded to it in a January 2020 keynote address on the urgent need for modern technology (2) – and it is understood from the ground up. Laboratory medicine professionals need to play a leadership role at this time of substantive transformation to ensure they are understood and heard. Motta believes that pathologists are still not necessarily represented at boards and in salient papers that describe workforce requirements in cancer pathways. And, where this is the case, the status quo needs to change. Pathologists who are singularly focused on addressing the immediate backlog of reporting also need to be given scope to play a crucial role in policy-shaping and decision-making at the national, regional, and local levels. They need to be able to change the conversation from one where technology is seen as the answer to one that seeks conscientious analysis of how we can improve pathways – before we use digital pathology as a tool to get there.

As Motta said, "Digital pathology is like any tool – including dynamite. In the right hands, it can be really useful. In the wrong hands, it can be quite destructive. Some managers think that installing digital pathology will solve all their problems without even modifying or adapting patients' pathways. That's not going to work."

Jane Rendall is Managing Director at Sectra, Stansted, UK.

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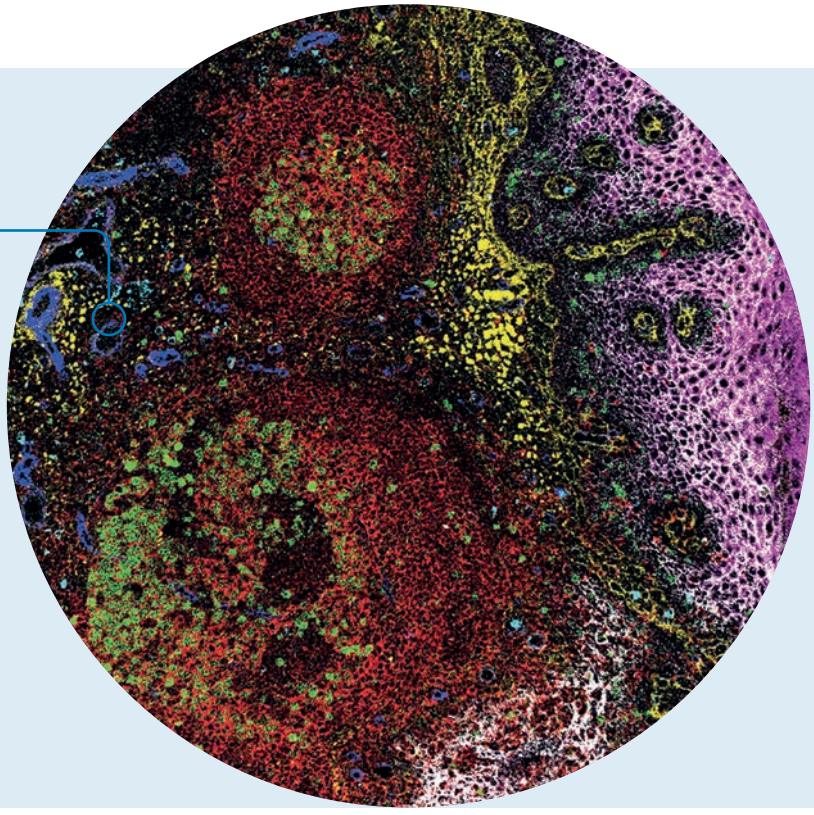

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Changing the Cancer Landscape

Sitting Down With… HRH Princess Dina Mired of Jordan, President of the Union for International Cancer Control and humanitarian and health activist

What brought you to cancer advocacy?

Growing up, we had one cancer hospital called the Hope Center (notice the omission of the word "cancer"). We walked past it every week, but we always ignored it because it was so easy to think that we'd never be the people who needed it. At that time, there was such a big taboo associated with cancer that nobody would talk openly about it. I never even considered cancer advocacy until our son, Rakan, was diagnosed with leukemia at the age of two. I didn't choose cancer; it chose my family.

Our battle with leukemia was long and challenging. After Rakan was treated in the UK, we moved to the USA and the cancer returned. My daughter donated her bone marrow and we were lucky – not only that she was a good enough match, but also that we had a strong support network. When our time in the USA came to an end, we dreaded going back to Jordan because cancer treatment and care was below average; the Hope Center was tantamount to a morgue. But we had to return – and that's when I was asked to join a new effort to transform the hospital. As a member of the Jordanian royal family, it's important for me to fulfil my title with public duty; that, and being the mother of a survivor, is why I joined the fight against cancer. I became Director General of the of the newly named the King Hussein Cancer Foundation.

What did you set out to achieve?

The main issue we, as board and management, had to resolve was that the hospital wasn't being managed effectively – despite having good infrastructure and fantastic, globally trained medical staff. It was a clear example of how cancer control is not just about the medical aspects, but also about how you manage the entire process. We followed the example of St. Jude Children's Hospital in the US, because they had a similar structure to the King Hussein Cancer Center, and we gained a Jordanian doctor from the National Cancer Institute who became, the first CEO of the King Hussein Cancer Center. I was Director General of the King Hussein Cancer Foundation for 15 years and, thanks to everyone's hard work and dedication, the King Hussein Cancer Foundation and the King Hussein Cancer Center became premier institutions of excellence not only in the region, but in the world.

What is the landscape like in Jordan now?

Not everyone in Jordan has the opportunity to receive care at the King Hussein Cancer Center. I would love to see more homogeneity in terms of cancer care quality across the country. The other thing I would like to see is improved screening. I was lucky enough to lead the Jordan Breast Cancer Program, which helped change perceptions of the disease among women in our country. Many women used to present in the late stages of cancer – but we've at least halved the number of women who present in stages III and IV. Unfortunately, people still have to pay for a test and not everyone can afford that. I would love to see a population-based screening program as part of an investment in prevention and early detection.

Tell us about your role at the Union for International Cancer Control (UICC)…

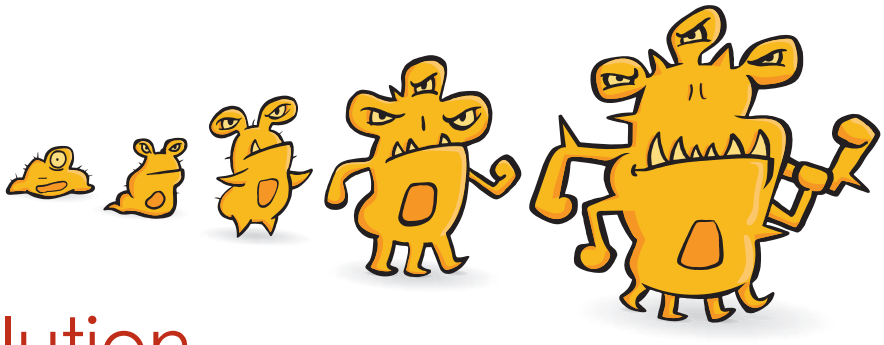
When I asked myself what I brought to the table before becoming President of the UICC – a position I am immensely proud to hold – two things stood out. I am probably the first non-medical person to be at the helm of such an institution but, given my own experiences, I felt that I truly understood the patient's perspective. I also come from the developing world, and I thought it was important for the UICC to have someone who had experienced the challenges and inadequacies faced by the places that they were aiming to help.

I love the organization's focus on partnerships and its lack of an ego. The UICC doesn't want to repeat things that others are doing; we see gaps and endeavor to address them. Although much has been done in terms of global advocacy, it doesn't mean anything unless it trickles down to the local level – and that's why we launched the City Cancer Challenge. This initiative encourages cities to form their own executive committees and influence cancer control in their local region. We facilitate and support these groups with strategies, action planning, and finance to help them identify gaps and implement their own managerial structure.

"The UICC doesn't want to repeat things that others are doing; we see gaps and endeavor to address them."

How can pathologists help?

Thanks to modern technology, pathologists can virtually train people and offer diagnostic opinions for cases around the world without having to travel. Professional bodies must help facilitate this by encouraging – and funding – global work. I also think it would be beneficial to construct an "essential laboratory list" to help labs in developing countries select the most appropriate equipment. I have seen countries waste huge amounts of money buying the most expensive machinery when the staff don't have the necessary skills and when outsourcing would be cheaper. In my opinion, the main stumbling block for cancer centers in the developing word is effective governance and management. And that's something we must – and can – improve across the board.



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