COLA'S 4th Quarter 2022

inSIGHTS

IN THIS ISSUE:

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LETTER FROM THE CHAIR

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Early this year, COLA received deemed status from CMS to accredit laboratories for the specialty of Pathology, including the sub-specialties of histopathology, oral pathology and cytology. Since then, COLA has extended our mission of promoting health and safety through accreditation and education further than ever before, contributing to the body of knowledge in Pathology through meaningful on-site surveys and educational courses and materials.

In this edition of inSights, we explore several technological advances in the field of cytopathology. Artificial intelligence and machine learning are gradually being adopted in cytopathology workflows and will likely see more widespread adoption as the underlying technology improves and becomes more accessible. Next-generation sequencing in molecular pathology is also becoming widespread as it allows for higher throughput and cost-effective DNA sequencing on cytological specimens. Together, these technologies stand to both increase efficiency in the cytopathology laboratory and improve treatment options for patients. Last but not least, we provide a summary of the evolution of cervical cancer screening since the 1950s.

We hope that you will find this edition to be useful and informative. We look forward to your feedback on these topics and to your suggestions regarding topics for future editions of inSights.

William E. Kobler, MD
Chair, COLA Board of Directors

ARTIFICIAL INTELLIGENCE AND CYTOLOGY

By: David Chhieng, MD, MBA, MSHI, MSEM, MLS, MD

Dr. David Chhieng is the Chief Medical Officer of COLA. Before he joined COLA, he was a Professor, the Director of Anatomic Pathology and Pathology Informatics, and Vice Chair of Clinical Operation, of the Department of Pathology at the University of Washington in Seattle WA. Prior to that, he was the Director of Cytopathology at Yale University and the Icahn School of Medicine at Mount Sinai. He is board certified in Anatomic and Clinical Pathology, Cytopathology, and Clinical Informatics. He has been a practicing surgical and cytopathologist for 20+ years and in a directorship position for 10+ years.

Introduction

Cytology, a subspecialty of pathology, refers to the microscopic study of cellular morphology and composition. In the 1920's, Dr. Papanicolaou invented the Pap test to screen for precancerous lesions on the uterine cervix in an attempt to prevent the progression to invasive cancer. Cytology has also been employed to evaluate lesions in the thyroid, breast, salivary glands, intra-thoracic and abdominal organs, fluids in various body cavities (pleural, peritoneal, pericardial and cerebrospinal) and other body sites and organs. When compared to histology, a key advantage of cytology is that it typically employs less invasive sampling procedures, which are relatively painless and low-cost, require less sophisticated equipment and do not require general anesthesia. One characteristic of cytologic evaluation is the identification of occasional abnormalities among the many thousands of cells scanned by cytologists. This manual process of scrutinizing every cell microscopically in search of scattered precancerous and cancerous cells can be very time consuming and tedious for cytologists.

Since the 1950s, researchers have attempted to develop automated methods for accurate and efficient evaluation of cytologic preparations.

Studies have shown that these automated cytology screening systems can improve efficiency while maintaining or exceeding accuracy when compared to manual cytology screening processes.

More recently, automation coupled with artificial intelligence (AI) has made substantial progress in the field of medicine, including in the specialty of pathology. AI is a branch of computer science concerned with the development of machines with the ability to mimic the human brain and perform complex tasks such as visual perception, decision-making and communication. It is a heterogeneous field that includes several sub-categories such as machine learning (ML), robotics and knowledge representation, among others. ML focuses on developing algorithms to represent the underlying patterns of data and make predictions, such as medical image interpretation and computer-assisted diagnosis in pathology. Contrary to rule-based AI systems, machine learning technologies develop algorithms by processing annotated examples, a process called training. One branch of the ML family that has been widely investigated for accurate and efficient cytologic screening is deep learning (DL). DL refers to the computational techniques that extract hierarchies of features without the need for a human to define features to extract. Application of DL in cytology has been widely investigated in various types of cancers, including cervix, breast, bladder, lung, oral cavity and thyroid.

This article aims to provide a review of the basic principle and role of AI, more specifically, DL, in cytology.

What is Deep Learning (DL)?

DL begins with the extraction of primitive features of the input images, followed by the combination of more specific features to form the complete image of the object. Subsequently, the more complex features of the image are extracted to develop a set of algorithms. The advantages of DL can be summarized into the following: 1) no need for feature extraction by the cytologists because of automatic data extraction, 2) no technical knowledge of image recognition or segmentation is required, 3) avoids the bias of cell selection, and 4) able to handle a massive amount of data from digital images or whole slide imaging. One tradeoff with DL is that it is essentially a "black box:" the means whereby the machines arrive at the answer for a given scenario remains uninterpretable by the user of the technology. Another tradeoff is that much larger datasets are typically required to develop such algorithms when compared to traditional manual rule-based approaches.

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DL can be classified into supervised and unsupervised learning. The former employs a training data set that has been accurately labeled by one or more experts. In contrast, unsupervised learning does not make use of a previously labeled dataset for training. The machine develops an algorithm to identify and classify unlabeled data without any human intervention. When compared to supervised learning, unsupervised learning makes it easier and faster to analyze complex data. In addition, it has the ability to identify previously undetected patterns. However, a considerable amount of data is often required for unsupervised learning. The most common type of DL currently employed in cytology is supervised learning.

Most deep learning methods use an artificial neural network (ANN) architecture. ANN is composed of layers of artificial "neurons" between input and output layers. Each layer includes one or more neurons linked with neurons in the previous and next layers. Among the various models of ANN, convolutional neural network (CNN) is regarded as the most successful DL architecture in image analysis. One major reason for using CNN is its ability to retain spatial information, which, in turn, allows CNN to measure each feature at every position in the image.

Digital Cytology

Digital pathology refers to the process of acquiring, managing, sharing and interpreting pathology information — including slides and data — in a digital environment. Laboratories have increasingly incorporated digital pathology in their cytologic practice for the past three decades. Initially, the focus was primarily on the screening of Pap smears. It was then followed by the application of digital pathology to allow real-time evaluation of specimen adequacy of fine needle aspiration biopsy, i.e. rapid on-site evaluation (ROSE), remotely.

A more recent development in digital pathology is the adoption of whole slide imaging (WSI) for primary diagnosis.

WSI is a digital scan of a glass slide, allowing the entire slide to be viewed on a computer monitor rather than through an optical microscope. Due to the increasing availability of WSI in the pathology laboratory and its ability to maintain the high complexity of the image including intact color information as well as the availability of information at multiple magnifications (e.g. ×4, ×20, ×40 objectives), DL and ANN can easily be applied to digitized whole slide images.

One major difference between cytologic and histologic slides is that the latter have a relatively uniform thickness (3-5 µm) with a relatively flat topography whereas the former consists of a non-uniform 3-dimensional arrangement of cellular and non-cellular components. Moreover, cytologic preparations often contain obscuring material, such as blood, inflammation, mucus or ultrasound gel/lubricant, that are not usually present in histologic sections. These unique features have a significant impact when applying WSI in cytologic preparations. One implication is the requirement of z-stacking (also called focus stacking), which consists of combining multiple images taken at different focal distances to provide a composite image with a greater depth of field (i.e. the thickness of the plane of focus) than any of the individual source images. This leads to increased image acquisition time and larger file sizes. The use of liquid-based preparation (LBP), which produces a more uniform monolayer distribution concentrated in a defined limited area of the slide, can partially alleviate this impact.

Currently, two WSI systems have been approved or cleared by the US Food and Drug Administration (FDA) for primary histopathologic diagnosis—one from Philips and the other from Leica Biosystems.

It is interesting to note that the FDA approval for these systems does not extend to cytologic preparations.

Validation by the laboratory is required prior to the implementation of any WSI system for patient testing to ensure the selected system performs as effectively or better than traditional light microscopy without any additional risks to patient safety. In addition, laboratories must continue to audit and evaluate the quality of digital images and diagnoses after adoption of WSI as part of a continuous quality management process. Last but not least, laboratories will be expected to invest substantially in their IT infrastructure, such as computer monitors of sufficient quality, devices with considerable storage capacity and network for efficient transmission of digital images between storage and individual viewing stations. Nonetheless, the incorporation of a robust digital workflow, particularly WSI, in cytology greatly facilitates the integration of AI, especially DL, into cytology operations.

CONTINUED ON PAGE 5 »»»

Artificial Intelligence and Cytology

Recent advancements in DL technology, along with the surge in the use of digital pathology, have spurred a lot of interest in applying AI to anatomic pathology (AP). The major benefit of AI is to save time and effort while improving diagnostic accuracy and interobserver agreement. Although the current spotlight of applying AI to AP has been focused mainly on histopathology, the initial focus of the earliest commercially available AI algorithms in AP was, as a matter of fact, gynecologic cytology. Given the tedious and repetitive nature of screening Pap tests and the sheer volume of tests, this was a logical choice. In the early 1990s, using neural network processing, the PAPNET system was designed to assist cytologists in identifying any abnormalities missed on prior manual microscopic examination of negative conventional Pap smears. Unfortunately, it failed to gain market acceptance because rescreening negative Pap smears did not provide a strong enough proposition to entice laboratories to acquire the PAPNET system. It is no surprise that subsequent attempts at applying AI to gynecologic cytology were instead designed to assist in primary screening of Pap tests. Currently, the ThinPrep Imaging System (Hologic, Marloborough MA) and Focal Point GS Imaging System (Becton Dickinson, Franklin Lakes, NJ) are two FDA-approved systems for use to assist the primary screening of ThinPrep and SurePath liquid-based gynecologic cytology, respectively. Besides addressing the need for automation and improved accuracy, there was additional reimbursement to the laboratory when using an automated system to assist in the primary screening of liquid-based gynecologic cytology, providing additional financial incentive. As a result, the adoption of these systems by cytology laboratories in the U.S. was more successful than their predecessor.

Over the past two decades, many investigators have applied AI and DL to non-gynecologic cytology.

Many of these attempts focus on the diagnosis of malignancy, the most crucial area of cytology. For example, in the late 1990s and early 2000s, the previously mentioned PAPNET system was applied on bladder washing samples to identify urothelial carcinoma, on sputum samples to detect lung carcinoma and esophageal and oral cytologic samples to detect squamous cell carcinoma. Some recent examples include the application of CNN models to thyroid fine needle aspiration cytology (FNAC) to diagnose papillary thyroid carcinoma (PTC) and to pancreatic FNAC to assign them into "suggestive/suspicious of malignancy" or "suggestive benign" categories, with high accuracy.

Besides accurately diagnosing malignancy, correct tumor subtyping is an essential component of cytology. The CNN model has been used in the classification of small-cell and non-small cell lung carcinomas in respiratory cytologic preparations and different subtypes of squamous cell carcinoma of the uterine cervix.

Risk stratification determines the risk level of patients suffering from diseases. A DL based model has been developed to assign risk stratification for urine cytology preparations by counting the number of atypical cells. Another example is the use of CNN to classify gynecologic cytology into 5 diagnostic categories according to the subsequent risk of developing invasive squamous cell carcinoma.

CONTINUED ON PAGE 6 »»

Challenges of Applying AI in Cytology

Despite great advancements and increasing applications of AI in cytology over the last few years, there are still quite a few challenges that need to be resolved. Firstly, the prerequisite of AI is the availability of large amounts of labeled data, which can be used to develop effective models and algorithms. Large-scale labeling, which requires manual delineation and annotation of regions of interest in WSI, can be a time-consuming task for cytologists. Unlike those of histopathology, public datasets of cytology are limited in number, cancer types and annotation types. In recent years, the introduction of more labeling-efficient learning, such as semi-supervised learning, allowing the use of both labeled and unlabeled data, has aimed to solve this problem. Many of the current AI algorithms and DL models have focused primarily on cellular components in the cytologic preparations.

Other components such as stromal elements and background matrix should be incorporated in the development of future algorithms and models.

Another related problem is how to transform DL research models into relevant clinical applications. The majority of DL models are validated on domain-specific datasets and often cannot achieve the same good performance with unseen, real-world data in a clinical setting. The term "overfitting" has been used to describe such a phenomenon. The diversity and complexity of clinical data relative to the training data as well as its incompleteness contribute to inferior performance in real-world clinical settings. Designing more robust architectures can make the AI models less dependent on data quality, potentially alleviating the issue of imperfect clinical data. On the other hand, it may be clinically harmful if the performance of an AI system surpasses that of humans. For example, an AI algorithm may be very sensitive, leading to increased detection of lesions with little or no clinical significance, which in turn may lead to unnecessary workup or intervention.

One critical success factor of the adoption of AI models in the clinical environment is the attitude of the cytologist. People, in general, are wary of change and reluctant to learn and adopt new technology. Instead of examining microscopic slides under a traditional light microscope, the cytologists will have to review digital images on digital monitors.

In addition, the cytologists would need to develop confidence in rendering their interpretations based on the quantitative and/or qualitative results provided by the AI-assisted diagnostic systems.

Therefore, the cytologists have to get accustomed to this new workflow.

Additionally, it is imperative that cytologists play an active role in the development of AI technologies for use in cytology to ensure that the AI algorithms that are being developed are clinically relevant. Another potential hurdle would be cytologists' distrust of the AI systems because many AI algorithms cannot be explained and/or interpreted at the level of human understanding, i.e., the "black box" problem.

Both regulatory and ethical requirements, such as patient consent, cybersecurity, data ownership and transfer to third parties such as commercial entities for the initial and ongoing algorithm development, etc., have not caught up with the advances in technology. Finally, proponents for the use of AI in cytology will need to justify the financial value of incorporating AI technology into routine cytology practice.

Conclusion

AI, specifically DL, is a rapidly emerging technology that can assist cytologists in improving the efficiency and accuracy of cancer screening and diagnosis. With the increasing adoption of digital cytology in clinical settings, AI and DL will likely play a significant role in augmenting cytology practice in the future. However, both regulatory and ethical requirements have yet to catch up with the advances in technology. It is important to remember that AI may be able to mimic human behaviors and thought processes, yet, many human qualities, such as critical thinking, interpersonal communication skills, emotional intelligence and creativity, cannot be perfected by machines.

NEXT GENERATION SEQUENCING AND CYTOLOGY

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Introduction

In recent years, the rapid advancement in our understanding of the molecular and genetic factors associated with the development and progression of various cancers has led to the development of therapies targeting specific molecular alterations. When compared to conventional chemotherapeutic agents, these new medicines improve disease responsiveness and overall patient survival while minimizing potential toxicity. Unlike the pancytotoxic effect of traditional chemotherapeutic agents that produce variable degrees of response within the general population, these new therapeutic agents do not offer any benefits unless the patients have demonstrated certain genetic and/or molecular alterations. In other words, the administration of these agents to individual patients is strictly dependent on the identification of their corresponding molecular targets in a particular patient, i.e., precision medicine. As a result, there is a need for accurate molecular/genomic testing to confidently identify specific cohorts of patients who will benefit from these new targeted therapies, if treatment is to be personalized.

Driven by the increasing popularity of precision medicine, patient selection for targeted treatment has evolved to include a large panel of markers aimed to personalize therapeutic regimens even further.

The use of multiple target genes makes conventional analyses such as Sanger sequencing and pyrosequencing labor-intensive, while the use of individual companion diagnostic kits is very expensive and the required quantity of cellular samples is not always available. Multiplexing assays based on single nucleotide primer extension using capillary electrophoresis or mass spectrometry can interrogate only a limited number of common variants and therefore, lack scalability to accommodate additional targets. On the other hand, next generation sequencing (NGS) testing offers a viable alternative at a lower cost, a reasonable turnaround time and a simple and efficient workflow.

Modern cytologists play a key role in integrating conventional cytology and novel molecular technologies, including NGS.

The use of cytologic samples for molecular testing, particularly NGS, offers several advantages. In many instances, cytology samples may be the only samples available for both morphologic evaluation and molecular testing because of simple, rapid, minimally invasive and flexible approaches to specimen collection.

In addition, fine needle aspiration (FNA) biopsy often yields a higher tumor fraction when compared to that of surgical biopsy specimens. The wide variety of cytologic preparations, such as direct smears, liquid-based preparations and cell blocks, provides many options for molecular testing. Because of the use of non-formalin fixatives, cytologic samples offer a better quality of nucleic acids than their histologic counterparts. Finally, because of the ability to perform adequacy assessment on-site, effective triage of FNA sample adequacy and quality can be performed to minimize non-diagnostic samples and potentially false-negative molecular results.

What is Next Generation Sequencing (NGS)?

The basic principle of an NGS platform consists of the use of a DNA polymerase module to add fluorescent-labeled nucleotides onto strands of DNA templates. The subsequent detection of the fluorescent signals by a highly sensitive capture system enables the visualization of the sequencing of DNA. This approach resembles that of real-time polymerase chain reaction (RT-PCR) and Sanger sequencing of DNA but the real power of NGS comes from its ability to offer massively parallel sequencing: the ability to simultaneously sequence billions of DNA fragments from thousands of genes or multiple tag patient samples mixed together in the same reaction.

CONTINUED ON PAGE 8 »»

The targets for NGS can range from the entire human genome, or whole genome sequencing; only the exomes, or coding regions of the genome; or smaller sets of specific targeted genes with known and actionable mutations. NGS can also be used for RNA sequencing to discover novel RNA variants and splice sites, or quantify mRNAs for gene expression analysis.

Generally speaking, NGS workflow consists of four phases: library generation, clonal amplification, massive parallel sequencing and data analysis. The first phase consists of random fragmentation of sample DNA into different lengths. The next phase is amplification where the DNA fragments are amplified into clonal groups. The third phase is the concurrent detection of thousands of base additions, also called massive parallel sequencing. The mechanisms employed depend on the platforms and include pyrosequencing, 454 sequencing, ion torrent semiconductor sequencing, sequencing by ligation, reversible terminator sequencing or nanopore sequencing. The final phase consists of data analysis which includes the identification of the nucleotides, a process called base calling, and the predicted accuracy of those base calls. The resulting sequencing data must be compared to a reference human genome with a number of available bioinformatic tools.

The final outputs would be either disease interpretation or actionable therapeutic outcomes.

Cytologic Sample Requirements for NGS

There are many factors that can affect the quality and quantity of a cytologic sample used for NGS. Some factors, such as the operator's skill and experience in procuring the samples, tumor characteristics such as size, anatomic site and the extent of necrosis and/or fibrosis, are beyond the control of the cytologists. Nonetheless, cytologists can and should play an important role in the implementation of a preanalytic process that enables the optimization of cytologic samples for both morphologic examination and NGS testing.

Types of Cytologic Preparation

Fixative selection is one of the most significant preanalytical factors capable of negatively affecting NGS results. Since histologic formalin-fixed paraffin-embedded (FFPE) tissues are often used to validate NGS, separate validation would not be needed for cytology FFPE cell blocks (CBs). However, the problem with CBs is that fixation in formalin, particularly if prolonged in time, may give rise to significant cross-linkage of molecules, resulting in suboptimal nucleic acid quality. In addition, initial H&E sections and those following those used for NGS testing should be evaluated, to ensure the adequacy of the sample used for NGS. It is important to point out that the process of CB preparation itself is far from standardized since there are a number of methods, both conventional and unconventional, used for making CBs.

Non-formalin cytologic preparations, such as direct smears and liquid-based cytology (LBC), provide a higher nucleic acid yield and quality than FFPE CBs. It is important to recognize that each of these preparations has its distinct advantages and disadvantages. For example, only direct smears can be assessed for adequacy by rapid on-site evaluation (ROSE) at the time of specimen acquisition. The downside of direct smears includes the limited number of available slides and the medico-legal implications of sacrificing archival slides. Additionally, direct smears would not be suitable for evaluating the consistency and reproducibility of NGS results among different laboratories owing to the unique and unreproducible nature of smears.

LBC has been widely accepted as a suitable substitute for morphologic evaluation in lieu of direct smears because it avoids the problem of suboptimal handling of the aspirated material by untrained healthcare providers. LBC slides or residual fluid are also emerging as a valid and alternative specimen option for NGS testing.

One major disadvantage of using LBC residual fluid is the impossibility of long-term storage.

Regardless of the type of non-formalin cytologic preparations used for NGS testing, additional separate validations would be needed before being considered suitable for NGS analysis in routine clinical practice.

Specimen Adequacy

Generally speaking, the higher the overall cellularity of the cytologic preparation, the higher the DNA yield will be, which in turn increases the likelihood of success of NGS. Equally important is the tumor cellularity/fraction: the proportion of tumor cells relative to the overall cellularity. The former relates to the analytic sensitivity of the NGS assays. Most NGS testing platforms have a sensitivity of 5-10%. This means that a minimum tumor cellularity of 10-20% is required to reliably detect a mutant allele in neoplastic cells within the background of wild-type alleles and minimize the potential of false-negative results. Tumor cellularity can be enhanced by tumor enrichment techniques, such as macro- or laser capture microdissection of direct smears and cell block preparations.

CONTINUED ON PAGE 9 »»»

Practical Considerations in Implementing NGS in Cytologic Practice

Clinical Application of NGS to Cytological Samples

Since cytologists often need to work with limited diagnostic material, 'do more with less' becomes a motto for cytologists. NGS offers cytologists a great opportunity to fully exploit the use of cytological samples to analyze a large number of gene regions and targets. One indication of NGS testing is to improve the sensitivity of detecting low-level true positive genetic mutations in pauci-cellular cytological samples with previously false-negative results using other direct sequencing platforms and/or those lacking diagnostic cells on morphologic evaluation. For example, 20% of pancreatic cyst fluid samples obtained through endoscopic ultrasound (EUS) guided pancreatic FNA were found by NGS to harbor a K-ras mutation, an indication of neoplastic process, despite the morphologic absence of diagnostic cells on corresponding cytologic preparations. Another indication of NGS testing is to refine uncertain cytologic diagnoses based on multigene molecular profiling. One notable example is the use of NGS gene panels to stratify equivocal thyroid cytological fine needle aspiration (FNA) samples into high- and low-risk categories. One disadvantage of NGS testing is the detection of mutations that may be of little or unknown clinical relevance. Last but not least, for patients with progressive malignant disease, serial molecular profiling of recurrent and metastatic lesions through FNA and NGS could be used to identify clonal evolution and the emergence of treatment-resistant clones.

Validation

As with any clinical assay, NGS-based assays need to be validated prior to use for patient care. The validation of NGS includes preanalytical, nucleic acid preparation, sequencing and bioinformatics steps. The Clinical Laboratory Improvement Amendments (CLIA) regulatory requirements require all laboratory-developed tests, i.e., non-Federal Drug Administration (FDA) approved/cleared tests to address accuracy, precision, reportable range, reference range, analytical sensitivity, analytical specificity, and any other parameter that may be relevant (e.g. carryover, specimen stability, etc).

The validation protocol should be developed and approved by the Laboratory Director before generating validation data.

Therefore, data collected during NGS test development are not part of the validation. However, such data can be used to set performance criteria for acceptance as well as determine the number and types of samples required for validation using samples of the type proposed for the assay so that test performance is representative of the intended testing population. For example, the validation protocol of an NGS test intended to detect known hotspot mutations using LBC residual fluid would include LBC residual fluid samples known to contain these types of mutations.

Choice of Gene Panels

The gene panels for NGS can range from small panels covering the hotspot regions of the most common actionable genes (up to 10-15 genes) to directly guide standard-of-care management, to intermediate-sized panels consisting of a collection of well-studied actionable genes that are commonly involved in several diseases (up to hundreds of genes) to allow enrollment of patients in clinical trials, and to larger, comprehensive panels covering the whole exome or genome. The latter approach is uniquely suitable for patients with undiagnosed diseases or patients with negative results using a limited, disease-focused panel. Cytologic preparations are particularly well suited for NGS testing with small gene panels since the latter requires a relatively small amount of DNA input.

Current Use of NGS Testing With Cytologic Samples

Because of the rapid advancement of molecular pathology and the ever-changing picture of available treatments, it would be beyond the scope of this paper to provide a comprehensive review of NGS testing in cytopathology of various cancer types.

However, this author would like to highlight two diseases, non-small cell lung cancers and thyroid cancers, which best demonstrate the interplay between cytopathology and NGS testing.

CONTINUED ON PAGE 10 »»

NGS Testing and Lung Cancer Cytologic Specimens

One of the most common applications of NGS testing in cytology is for predictive biomarker testing in lung cancer. This can be attributed to the recent and ongoing developments in the treatment and management of lung cancer resulting in growing complexity of molecular testing requirements. In addition, many patients with non-small cell lung carcinoma (NSCLC) are diagnosed at an advanced and inoperable stage and cytology is frequently the only sample available for NGS testing. NGS testing can be performed on all cytologic preparations obtained from either primary or metastatic lung cancers. The rejection rate for NGS-based predictive marker testing using cytologic preparations has been reported to be less than 5%.

The current guidelines recommend testing of all advanced-stage patients with non-squamous morphology NSCLC for, at a minimum, Epidermal Growth Factor Receptor (EGFR), Anaplastic Lymphoma Kinase (ALK), ROS Proto-Oncogene 1 Receptor Tyrosine Kinase (ROS1) and V-Raf murine sarcoma viral oncogene homolog B (BRAF) to guide standard-of-care management. C-MET Proto-Oncogene (MET), RET Proto-Oncogene (RET), Neurotrophic Tyrosine Receptor Kinase (NTRK) 1/2/3 and Human Epidermal Growth Factor Receptor -2 (Her2) should be included in any expanded panel, in case there is access to the corresponding targeted drugs via clinical trials or compassionate use programs. In the past decade, expression level of Programmed Death-Ligand 1 (PD-L1) via immunocytochemistry (ICC) has been added to the current predictive marker panel for advanced non-small cell lung cancer.

Although alcohol-fixed cytologic samples are preferred for NGS-based testing, concerns about false-negative results have been raised over the use of alcohol-fixed samples for evaluating the expression of PD-L1 using ICC because of the likely loss of PD-L1 expression with alcohol fixation. This observation highlights the need for careful preanalytic planning of fixation as well as handling and processing of cytologic samples.

NGS Testing and Thyroid FNA Specimens

Thyroid FNA is the diagnostic procedure of choice when investigating patients with thyroid nodules. In addition, rapid on-site evaluation is often performed to ensure adequate specimen samples and allow triage for any ancillary testing needs. Although thyroid FNA is accurate and cost-effective, in up to 25% of thyroid nodules a definitive morphologic diagnosis cannot be rendered.

They are consequently classified as indeterminate cytologically.

There have been many efforts in developing molecular tests to triage thyroid FNA with indeterminate cytologic classifications to improve the accuracy of thyroid FNA and refine management decisions. The various genetic alterations that have been identified and chosen as molecular markers in thyroid carcinogenesis include mutations in BRAF, Harvey rat sarcoma viral oncogene homolog (H-ras), neuroblastoma ras oncogene viral homolog (N-ras), Kirsten rat sarcoma viral oncogene homolog (K-ras); translocations such as rearranged during transfection/H4 gene fusion (RET/PTC1), RET/PTC3 (formed by fusion with ELE1) and PAX8-PPARγ (paired box gene 8-Peroxisome proliferator-activated receptor gamma).

Other genetic mutations reported in thyroid cancers include EIF1AX (Eukaryotic translation initiation factor 1A, X-chromosomal), TERT promoter (promoter of Telomerase reverse transcriptase), DICER1 (encoding an endoribonuclease) and GNAS gene (encoding for adenylate cyclase-stimulating G α-protein).

Hence, panels of these molecular markers and others in varying combinations have been devised to improve the accuracy in the characterization of indeterminate thyroid FNA.

Both laboratory-developed and commercially available NGS-based assays, designed for sensitive detection of various thyroid cancer-related gene mutations and rearrangement, have been increasingly applied to further stratify the risk of harboring a thyroid malignancy (ROM) using samples obtained during thyroid FNA. A clinical validation study reported that ThyroSeq version 3, a commercially available NGS-based assay to detect over 100 genetic alterations, had a sensitivity of 94% and negative predictive value of 97% in its validation studies, therefore accurately "ruling out" thyroid malignancy in patients with indeterminate thyroid cytology. By ruling out thyroid malignancy based on NGS testing, the patients can be followed conservatively with periodic ultrasound examinations, thus avoiding unnecessary surgery such as diagnostic lobectomy.

Conclusion

NGS is a modern tool that has revolutionized how molecular testing is performed. It has enabled molecular laboratories to take advantage of cytologic preparations with relatively low nucleic acid inputs to evaluate vast arrays of different molecular and genetic alterations simultaneously for diagnostic and predictive purposes. Preanalytic factors such as fixation and types of preparation can have a substantial impact on the quality of the cytologic samples used for NGS-based assays. Cytologists should understand the requirements and be actively involved in the procurement and preparation of high-quality cytologic samples for NGS testing.

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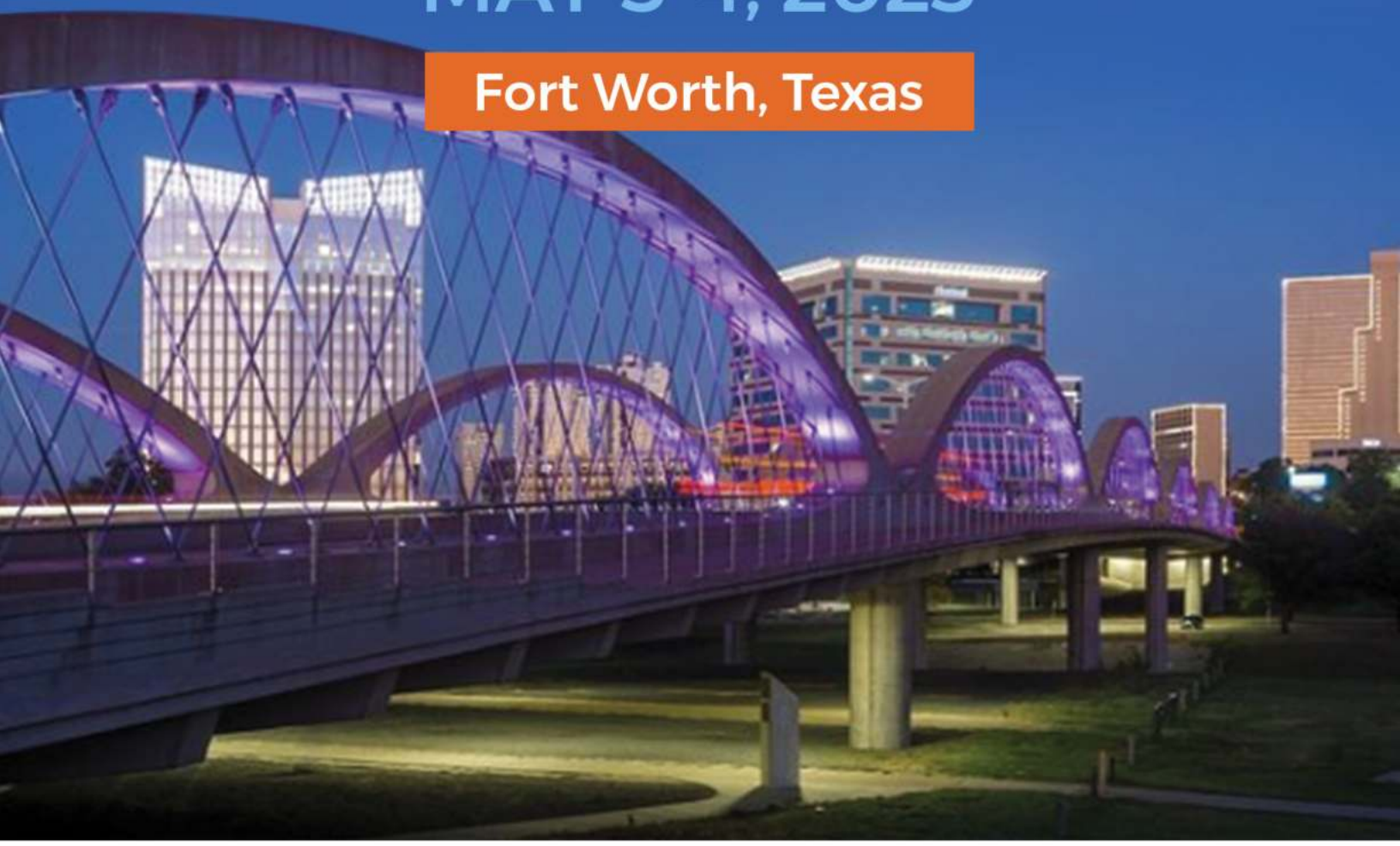

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EVOLUTION OF CERVICAL CANCER SCREENING

The First Successful Cancer Screening Strategy

By: David Chhieng, MD, MBA, MSHI, MSEM, MLS, MD

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Invasive cervical cancer refers to cancer originating from cells lining the uterine cervix, which connects the vagina to the body of the uterus. It is the fourth most common malignancy among women worldwide. Cervical cancer screening, consisting of early detection and treatment of preneoplastic lesions, is instrumental in reducing the incidence and mortality rate of invasive cervical cancer. As our understanding of cervical cancer advances, so do the strategies used in cervical cancer screening. This article is to provide a review of the evolution of cervical cancer screening.

Epidemiology of Cervical Cancer

When cervical cancer was first discovered in the late 19th and early 20th century, cervical cancer was both endemic and epidemic. It was a common cause of death for women in high-income countries, including the United States, until the introduction of widespread cervical cancer screening programs in the 1950s. Since then, both incidence and mortality rates of cervical cancer in the U.S. have declined significantly by more than 70%.

Worldwide, cervical cancer is the fourth most frequent of all female malignancies with 90% of the cases occurring in low- and middle-income countries. Within the U.S., it is estimated that about 13,000 new cases of cervical cancer are diagnosed and about 4,000 women die of this cancer each year. Global rates are considerably higher: approximately 600,000 new cases of cervical cancer are diagnosed and 300,000 women die of the disease annually in developing countries. These disparities are primarily due to the lack of well-organized screening programs as well as other effective interventions such as vaccination against human papillomavirus (HPV).

Biology of Cervical Cancer

The epithelial lining of the uterine cervix is made up of two embryonically distinct cell types: 1) the ectocervix, extending into the vagina and consisting of nonkeratinized stratified squamous epithelium and 2) the endocervix, leading into the uterine corpus and covered by columnar glandular cells. The junction of the ectocervix and endocervix is called the squamocolumnar junction which migrates towards the uterus with age, replacing columnar cells with stratified squamous epithelium. Due to the rapid turnover of cells, this so called "transformation zone" is very susceptible to carcinogens and neoplastic transformation, i.e., the beginning of cervical cancer. Fortunately, this process occurs gradually and over a long period of time. Furthermore, for the majority of women, these precancerous cells often regress spontaneously without intervention. For a small number of patients, precancerous lesions will persist and eventually progress into invasive cancer. The goal of cervical cancer screening is to detect and treat cervical precancerous lesions before they develop into invasive cancer, leading to improved patient outcomes.

The Pap Test

The Conventional Pap Test

Before the development of modern cervical cancer screening tools, pathologists relied on the examination of tissue biopsies obtained from a visible lesion when the cancer was quite advanced. In 1916, Dr. Georgios Papanicolaou, a Greek immigrant, described the cytologic patterns and physiologic changes of exfoliated vaginal cells obtained from guinea pigs while working as an assistant professor in the Anatomy Department of Cornell University Medical College.

In 1920, he hypothesized that similar microscopic changes could be appreciated in humans and began to focus on evaluating samples from human volunteers.

CONTINUED ON PAGE 13 »»

In 1925, after coming across a sample of exfoliated vaginal cells obtained from a woman with undiagnosed cervical cancer, he began to study and characterize cervical and vaginal cells from patients with cervical cancer. In 1928, Dr. Papanicolaou presented his research, which was rejected by the pathologists of the day due to errors and misspellings as well as the commonly held belief that exfoliated cells could not be used to diagnose cervical cancer.

Despite the rejection, Dr. Papanicolaou continued his research on cervical cancer and further developed his screening method.

In 1939, he collaborated with gynecologist Dr. Herbert Traut on a clinical trial to evaluate the use of Papanicolaou's screening test on more than 3,000 women. Their findings, which included photographs and descriptions of normal and cancerous exfoliated cells, were published in the American Journal of Obstetrics and Gynecology in 1941. The researchers diagnosed over 100 cases of cervical cancer, nearly all of which were not detected by visual inspection of the cervix and would not have been discovered without evaluating the exfoliated cells microscopically. They proposed that their simple and inexpensive technique could be used to screen large numbers of women for cervical cancer, especially early stage cancers amenable to effective treatment.

This technique was further improved by Dr. J Ernest Ayre, a Canadian gynecologist, who introduced a scraping method to obtain cells directly from the cervix with a wooden spatula. This new approach allowed for easier collection of more cells, offering greater sensitivity over Papanicolaou's vaginal pool method and the use of glass pipettes. Wooden spatulas have largely been replaced by broom/brush type devices for collecting cervical cell samples. The sample is "smeared" onto a glass slide, which is then rapidly sprayed with or immersed in a fixative solution to preserve the cells and subsequently sent to the laboratory for staining and examination by a cytologist. This method of preparing gynecologic cytology specimens is usually referred as the "conventional" method.

After the publication of his 1943 monograph that included detailed drawings of normal and abnormal cervical cells, Papanicolaou's technique, or the Pap test, gradually gained acceptance until it became a regular part of women's annual health care screenings nationwide. The Pap test is now considered the most successful cancer screening test. After the widespread adoption of the Pap test, the incidence and mortality rates of cervical cancer deaths dramatically decreased over the next 50 years.

Like all screening tests, conventional Pap tests have limitations.

One major concern is the relatively low sensitivity (55-65%) of a single Pap test performed at one point in time. Repeat screening at regular intervals, typically annually, compensates for such limitations. One contributing factor to the low sensitivities of conventional Pap tests is sampling error because only a small fraction of the cellular material collected from the cervix is transferred to the glass slide for evaluation. Additionally, the process of microscopic evaluation of conventional Pap tests by trained cytologists to identify a few abnormal cells among thousands of normal cells is labor-intensive and subjective. Uneven cellular distribution, overlapping cells, as well as obscuring inflammation and blood can further hinder the screening process.

Liquid-Based Cytology

Liquid-based cytology (LBC), first introduced in the late 1990s, aims to overcome some limitations of the conventional Pap test. Instead of smearing collected cervical cells onto a glass slide, the sampling device used for LBC is vigorously rinsed in a vial of preservative fluid. This results in a suspension of cells that is subsequently used to deposit a single layer of cells on the slide. There are several advantages LBC offers over conventional preparation. LBC ensures more and better preservation of the cells in the collection media. In addition, the slide preparation process results in a more even cellular distribution with minimal overlapping (i.e., a monolayer) and reduces the effects of contaminants such as blood cells, inflammation and mucus.

Although LBC does not offer superior sensitivity compared to conventional preparation for the detection of high-grade squamous intraepithelial lesions (HSIL) and cancer, it results in more positive findings, and therefore higher positive predictive value. Additionally, cytologists receive significantly fewer unsatisfactory slides with LBC than with conventional preparation. Another key added benefit of LBC is that the residual specimen can be retained and used for additional testing, such as HPV testing, eliminating the need to collect a separate sample during the same or separate visit. Currently, LBC has largely replaced conventional Pap tests in the U.S. and many other countries.

CONTINUED ON PAGE 14 »»»

Semi-automated Screening Technologies

Screening of Pap test by cytologists is labor-intensive and subjective. To improve screening efficiency and accuracy of Pap test screening, a number of proprietary computer technologies have been developed to automate the manual screening process. There are two major approaches: 1) those that perform primary screening without cytologist interaction and 2) those that require cytologist's interaction by directing their focus on areas of the slide deemed not normal by the computer. There are currently two commercially available imaging systems approved for use in gynecologic cytology by the Food and Drug Administration (FDA) in the U.S. Both of these systems fall into the second category. When compared to manual screening, conflicting data have been reported about the performance, impact on process and cost-effectiveness of these semi-automated screening instruments.

Human Papillomavirus

Though gynecological cytology remains an important tool in cervical cancer screening strategy, focus has been shifted to incorporate HPV-based testing to cervical cancer screening strategy in the past 2 decades. The link between HPV and cervical cancer was first discovered by Hans zur Hausen in the 1980s. In the mid-1990s, the World Health Organization recognized that infection with certain HPV strains is the most important risk factor in the pathogenesis of cervical cancer.

Natural History

HPV infection is ubiquitous; almost all sexually active individuals will be infected with HPV at least once in their lifetime.

Fortunately, cervical cancer is relatively rare and there is a long latency period between the initial HPV infection and the eventual development of invasive cervical cancer. Moreover, the majority of HPV infections are either asymptomatic or associated with low-grade squamous abnormalities, i.e., low-grade squamous intraepithelial lesion (LSIL) and atypical squamous cells of undetermined significance (ASC-US). Most LSIL and ASC-US, whether associated with symptoms or not, regress spontaneously and do not progress to cancer, especially in patients under the age of thirty. In contrast, HPV infections that persist over time and those associated with HSIL are associated with the integration of HPV genomes into the host cells and an increased risk of developing cervical cancer.

Among the 200+ genotypes of HPV that have been identified, over 40 genotypes affect the genital tract. Those are further classified into high- and low-risk types. Low-risk HPV (lrHPV) types, such as 6 and 11, are associated with sexually transmitted anogenital warts (condyloma acuminata). On the other hand, high-risk HPV (hrHPV) types are linked to precancerous and cancerous cervical lesions, with HPV 16 and 18 accounting for 60 and 10 percent of invasive cervical cancers, respectively.

HPV-based Testing

While Pap tests can allow cytologists to infer the presence of HPV infection based on morphological abnormalities, the emergence of molecular techniques allows the identification of HPV infections, classification of infection as high- or low-risk types and determination of the specific genotypes in cervical samples. The ability to genotype, especially HPV 16 ad 18, allows for the identification of women at the greatest risk of developing cervical pre-cancerous and cancerous lesions.

Since the late 1990s, the FDA has approved 5 testing modalities for the detection of HPV in gynecologic cytological specimens. They are Hybrid Capture 2 (Qiagen, Germantown MD), Cervista (Hologic, Marlborough, MA), Cobas 4800 (Roche, Basel, Switzerland), Aptima (Hologic), and Onclarity (Becton Dickinson, Franklin Lakes, NJ). The Hybrid Capture 2 (HC2) HPV DNA assay was cleared by the FDA for triaging women with equivocal cervical cytology in 1997. It is a nucleic acid hybridization assay that uses signal amplification for the qualitative detection of 13 hrHPV types in cervical specimens. It can be used for both ThinPrep and SurePath Pap tests. Limitations include cross-reactivity with many lrHPV types and the lack of an internal control; the latter implies that false-negative results due to test failures would be reported as negative. HC2, as the only FDA-cleared HPV assay, served as the gold standard for cervical cancer screening until the early 2010s when the FDA approved other HPV tests.

The Cervista HPV test was first approved in 2009 by the FDA for use with ThinPrep specimens.

It uses signal amplification for detection of specific nucleic acid sequences targeting the L1, E6 and E7 genes. It detects the presence of 14 hrHPV types. However, it cannot distinguish the specific hrHPV type(s) present. The Cobas HPV Test is a qualitative test that amplifies target L1 DNA by polymerase chain reaction (PCR) and nucleic acid hybridization for the detection of 14 hrHPV types. It also specifically identifies HPV 16 and 18. Cobas HPV assay was approved for use on both ThinPrep (2011) and SurePath (2016) specimens. The Aptima HPV Assay was FDA approved in 2011 for use with ThinPrep specimens. It is a nucleic acid amplification test for the qualitative detection of E6/E7 viral messenger RNA (mRNA) from 14 hrHPV types. Since it does not include genotyping, a separate test must be performed to specifically identify types 16 and 18. The Onclarity HPV Assay is the latest FDA-approved HPV assay for SurePath Pap tests. It employs target amplification of DNA by real-time PCR and nucleic acid hybridization for the detection of 14 hrHPV types with extended genotyping for individual detection of types 16, 18, 31, 45, 51 and 52.

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It has also been evaluated for HPV-vaccinated women, with lower sensitivity (80%) and higher specificity (52%) in these women when compared with unvaccinated women (100% and 46%, respectively). Although these HPV assays differ in methodology, target and analytic cutoffs, they are all interpreted as either positive or negative for hrHPV and offer comparable clinical implications for patient management.

Role of HPV testing in Cervical Cancer Screening

HPV testing has been used to identify women at increased risk of developing high-grade precancerous and cancerous cervical lesions since the early 2000s. When HPV testing was first being considered as one of the cervical cancer screening tools, it was approved for triaging women with minimally abnormal cervical cytology; namely, atypical squamous cells of undetermined significance (ASC-US). This approval was based on the findings of the National Cancer Institute (NCI)-sponsored ALTS trial (the Atypical Squamous Cells of Undetermined Significance – Low-Grade Squamous Intraepithelial Lesion Triage Study). Later, HPV testing was also recommended as a follow-up for women who were treated for a precancerous cervical lesion.

In 2012, there were several major developments in the U.S. cervical cancer screening guidelines.

First was the recommendation of co-testing with hrHPV and cytology every 5 years for routine screening of women aged 30-65. The use of co-testing allows for an extended screening interval and provides better sensitivity for detecting high-grade precancerous cervical lesions than cytology alone. If hrHPV testing was not available, the guidelines recommended Pap tests every three years as an acceptable alternative. Another major development was the recommendation of the identification of HPV genotypes 16 and 18 for women who have discordant co-testing results with normal cytology and positive hrHPV results. A positive test for either HPV 16 or 18 was recommended to be followed up with colposcopy. On the other hand, a negative result would have the patient return for repeat co-testing in one year.

Because of its superior negative predictability over Pap tests, cervical cancer screening has shifted toward primary HPV testing. In 2014, the FDA approved the use of Cobas HPV test for primary screening based on evidence from 3-year follow-up data of the 47,000 women reported in the industry-sponsored ATHENA study. In 2018, the U.S. Preventive Services Task Force (USPSTF) included the option of using primary HPV testing alone for screening women aged 30-65 years every five years in its guideline. This marked the first time in the U.S. that Pap tests would not be included as the primary screening tool for cervical cancer.

This change has triggered strong pushback from some experts. These dissenting experts argued that HPV testing alone had higher rate of false positives, resulting in unnecessary colposcopic examination and biopsy. In addition, they also noted that cervical cancer screening in the U.S. tended to be opportunistic without reliable follow-up. Furthermore, they also argued that HPV testing did not identify all women with precancerous and cancerous lesions and that the research supporting the use of primary HPV testing was largely conducted in Europe and Australia among cohorts including few women of color and using different HPV tests than those used in the U.S. Despite such pushback, in 2020, American Cancer Society updated its guidelines and recommended primary HPV testing alone every 5 years for every woman with a cervix between aged 25 to 65 years, with the option of performing HPV/Pap co-testing every 5 years or a Pap test alone every 3 years if an FDA-approved primary HPV test is not available. A year later, the American College of Obstetricians and Gynecologists, the American Society for Colposcopy and Cervical Pathology and the Society of Gynecologic Oncology endorsed the 2018 USPSTF cervical cancer screening recommendations. Currently, there are two hrHPV tests, Cobas and Onclarity, approved by the FDA for primary screening of cervical cancer in individuals aged 25 years and older.

Future Directions of HPV-based Testing

Despite the demonstrated efficacy and efficiency of primary hrHPV testing and the updated cervical cancer screening guidelines, adoption of this screening strategy in the U.S. has been slow.

CONTINUED ON PAGE 16 »»

This can be partly attributed to the limited availability and accessibility of FDA-approved tests and the significant investment for laboratory to switch to an FDA-approved primary HPV testing platform. With the continuous improvement of HPV vaccination rates nationwide, HPV prevalence is expected to continue to decrease. The latter could prompt future changes to screening guidelines, such as raising the screening age to 25 years.

Another possible direction would be the use of HPV self-sampling. Instead of visiting a provider for a pelvic examination where specimens would be collected, patients themselves could collect their own cervicovaginal HPV DNA samples and send them directly to a laboratory. This approach has gained attention for its potential to increase uptake of cervical cancer screening, especially in low- and middle-income countries. Although recent research has demonstrated its efficacy and utility, HPV self-sampling is still under investigation in the U.S.

Emerging Technologies for Cervical Cancer Screening

Although HPV testing is gradually replacing cytology as the main strategy for cervical cancer screening, the specificity of HPV testing is considerably lower than that of cytology, resulting in an increased rate of colposcopy after positive results. To minimize unnecessary colposcopic procedures, it would be ideal to develop an efficient triage method for HPV testing. As a result, new biomarkers, such as p16/Ki-67 dual staining and DNA methylation, have been developed to triage patients with HPV-positive results.

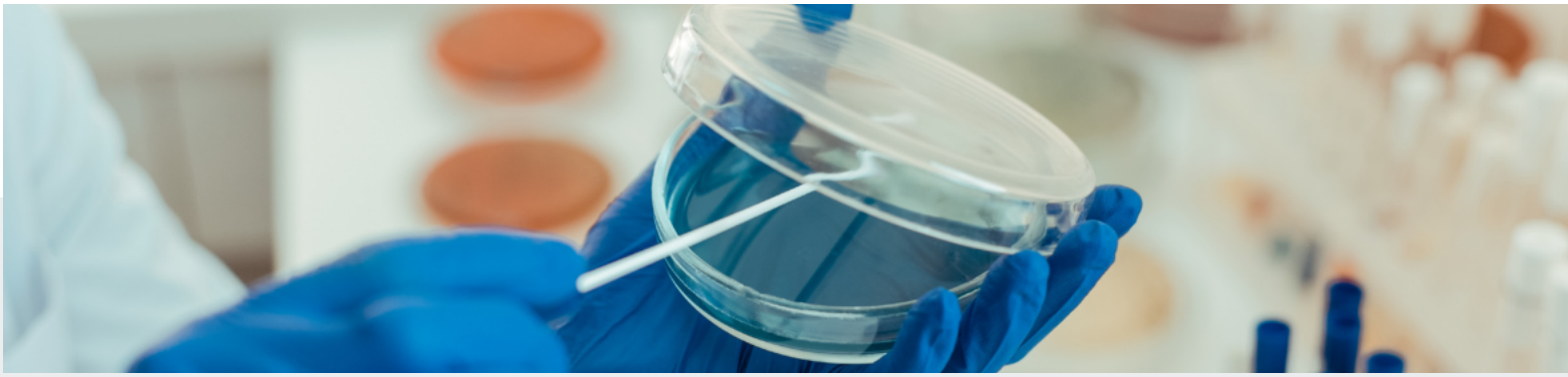
p16/Ki-67 Dual Staining

Neoplastic transformation as a result of persistent HPV infection results in the concurrent expression of p16, a tumor suppressor protein, and Ki-67, a proliferation marker, in the same cell. These markers are mutually exclusive in non-neoplastic cells, including those that are transiently infected by HPV. Dual staining of p16/Ki-67 can be accomplished by performing immunocytochemistry with antibodies against p16 and Ki-67 on cytologic preparations. It has been demonstrated that both the sensitivity and specificity of p16/Ki-67 dual staining to detect cervical intraepithelial lesions (CIN 2+/CIN 3+) were higher than those of cytology for triage of HPV-positive women. Currently, CINtec Plus (Roche) is the only FDA approved triage test that uses dual biomarker technology to simultaneously detect p16 and Ki-67 using cytologic preparations in women with HPV-positive results.

DNA Methylation

DNA methylation refers to the process by which methyl groups are added to DNA molecules, resulting in possible changes in the activity of a DNA segment without alteration to the sequence. Aberrant DNA methylation patterns can lead to defective gene expression, faulty condensation and chromosomal instability and has been recognized as an important element of carcinogenesis. In relation to cervical carcinogenesis, aberrant DNA methylation is associated with persistence of hrHPV infection, the severity of preinvasive neoplastic cervical lesions and the risk of developing invasive cancer. Presently, there are more than 80 methylation patterns that are frequently encountered in cervical cancer and one or more of them can serve as possible biomarkers to identify HPV-positive women at risk of developing invasive cervical cancer. It has been proposed that a DNA methylation assay could be used as a primary screening screen or for triaging HPV-positive women who may be at risk of developing or harboring invasive cancers. Compared with cytology, DNA methylation is objective and decreases interpretation errors.

Additionally, automation of the process is possible and both provider-collected and self-collected samples can be used.



Summary

Thanks to the successful screening strategy, cervical cancer is one of the most preventable cancers today. For decades, the Pap test was the standard for cervical cancer screening. With the advance of our understanding of the causative role of HPV in cervical carcinogenesis, there was a shift in the screening paradigm to HPV-based screening tests, beginning with triaging women with low grade squamous abnormalities to HPV/cytology co-testing and more recently, to the use of primary HPV testing alone. Furthermore, emerging technologies, such as p16/Ki-67 dual staining and DNA methylation, have been developed to further improve our abilities to accurately triage HPV-positive women who are at risk of developing cervical cancers.

One thing is certain. Change is inevitable.

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