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PATHOLOGY & LABORATORY MEDICINE NEWSLETTER

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Recent Advances in Cervical Cancer Screening
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- Limitations in Current Molecular Diagnostics in Predicting Risk of Cervical Cancer.
- A New Dimension to Predicting Cervical Cancer: New high through-put, flow cytometry-based HPV E6 & E7 mRNA assay developed for cervical cancer screening cytology specimens performed in less than 3 hours.
- The level of E6 and E7 gene expression potentially a functional discriminator between high-risk and low-risk HPV infections.

Laboratory Update:

New ("Old") PTH (Parathyroid Hormone) Assay at Stanford Clinical Laboratory

Q&A: B-type natriuretic peptide: NT-proBNP vs. BNP Assays

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Recent Advances in Cervical Cancer Screening

A New Dimension to Management of Patients from Stanford Clinical Laboratory

Cervical Cancer and Human Papillomavirus.

Cervical cancer is one of the most common malignant diseases among women worldwide⁽¹⁾. It is estimated that there are more than 50 million Papanicolaou (Pap) smears performed each year in the United States with more than 5% reported as abnormal. Despite the success of the Pap smear, limitations of cytology which include subjectivity, variability and failure to detect cases among borderline smears have slowed further reduction of cervical cancer cases.

More than 99% of cervical squamous cell carcinomas are due to infection and subsequent transformation of squamous cells by oncogenic genotypes of human papillomavirus (HPV). Studies have also implicated HPV in the pathogenesis of adenocarcinoma of the uterine cervix. The detection of HPV in the majority of cases of cervical neoplasia is considered evidence implicating the etiological role of HPV⁽¹⁾. This association is consistent and specific to a limited number of HPV types including 16, 18, 31, 33 and 45. HPV-16 alone accounts for 50% of the cases.

Limitations in Current Molecular Diagnostics in Predicting Risk of Cervical Cancer.

Most HPV DNA detection assays such as type-specific polymerase chain reaction (PCR) and Hybrid Capture II (Digene, Gaithersburg, MD) detect the presence of HPV L1 DNA, which is a region of the HPV gene that is expressed late and responsible for viral structural proteins. Previous studies have shown that HPV DNA testing is more sensitive than cervical cytology in detecting cervical intra-epithelial neoplasia grade 2 (CIN 2)

and CIN 3^(2,3). Until the wide acceptance of HPV DNA testing using the Hybrid Capture (HC) product, there was no consensus as to the appropriate management of the estimated 3 million women with low-grade squamous intraepithelial lesions (LSILs) or equivocal cytologic abnormalities referred to as ASCUS or atypical squamous cells of undetermined significance.

Based on the study of Solomon as well as other investigators, triaging ASCUS using the HC HPV DNA assay has become a routine approach to the management of women with ASCUS in the United States. While triaging ASCUS patients using the HC HPV DNA test is considered cost-effective compared to a protocol involving re-visits to a physician for repeat PAP testing and colposcopy, it has been estimated that the HC HPV DNA testing triaging of ASCUS cytologic diagnosis would result in a little over half of the women being referred to colposcopy⁽³⁾. In Solomon's study, HC HPV DNA testing exhibited a sensitivity of 96% compared with 83% for liquid-based PAP with a NPV for a HPV/PAP of 99.2% for CIN 3, which is consistent with a sensitive triage method for detection of CIN 2, and CIN 3. However, the **positive predictive value (PPV)** was **very poor** ranging from 10.0% to 19.6% for CIN3+ and CIN2+, respectively. The poor specificity of current HPV assays including HC HPV DNA testing and PCR can be understood by the fact that these assays detect L1 DNA from oncogenic types despite the fact that in only a minority of women infected with oncogenic types of HPV will the infection progress to cancer⁽⁴⁾. Detection of HPV L1 DNA is not necessarily consistent with carcinogenesis of the cervix. Behavior factors such as smoking have been shown in case control studies to correlate with

“(HPV) E6 & E7 as a potential adjunct to the Pap Smear.”

increased odds ratio; furthermore, host immune factors play a role in viral integration into host genome and disease progression.

A New Dimension to Predicting Cervical Cancer.

The integration of the HPV viral DNA into the cell DNA can result in an overexpression of E6 and E7 proteins. **In HPV-associated lesions, transcriptional activity is always present.** The expression of the HPV E6 gene promotes premature degradation of the tumor suppressor gene p53, and the HPV E7 gene associates with the tumor suppressor gene pRB. This association results in the up-regulation of an E2F-like transcription factor, promoting progression of the cell cycle through the G1/S phase. The RB gene product represses expression of p16, which is a marker strongly associated with cancer and its precursor. With inactivation of p53 and pRB, there is nothing to stop the cell cycle at the G1-S checkpoint.

The molecular switch for the development of cervical intraepithelial neoplasia and, ultimately, cervical cancer is the continued expression of the E6 and E7 genes of oncogenic HPVs. This initiates a cascade of events that results in the loss of tumor suppressor activity and cell cycle dysregulation.

Kraus, et al was able to demonstrate the presence of E6 and E7 mRNA for HPV types 16, 18, 31, 33 and 45 in 187 of 204 (92%) fresh frozen biopsies using a real-time multiplex NASBA assay, PreTect HPV-Proofer⁽⁴⁾. At Stanford, Narimatsu and Patterson went one step further by demonstrating that their high throughput, flow cytometry-based HPV E6 and E7 mRNA assay had higher sensitivity and specificity than HC HPV DNA detection with respect to Pap smears in women undergoing routine cervical cytology at multiple sites⁽⁵⁾. The assay that was developed can be performed in less than 3 hours directly from liquid based cervical cytology specimen. In their assay, E6 and E7 gene expression assessed by fluorescence in situ hybridization (FISH) was found to shown to be a reliable way of discriminating between high-risk and low-risk HPV infection. Narimatsu and Patterson were able to demonstrate that *E6 and E7 gene expression pattern changes with the severity of the lesion*; the level of E6 and E7 gene expression is increased in high-grade lesions compared with low-grade lesions, making the level of E6 and E7 gene expression potentially a functional discriminator between high-risk and low-risk HPV infections. The Stanford study demonstrated the usefulness of the assay in detecting abnormal cervical cytology ranging from atypical cells of undetermined significance (ASCUS) to invasive cervical cancer. Overall, HPV fluorescence in situ hybridization for E6 and E7 mRNA demonstrated 83.3% sensitivity and 91.3%

specificity for high-grade squamous intraepithelial lesions compared with the Pap test in 231 liquid-based cytology samples from 2 cohorts.

HPV E6/E7 as a Potential adjunct to the Pap Smear.

Although HPV DNA detection is biologically relevant, the detection of high-risk HPV DNA loses power because it detects a molecular event too early in the process. If disease in 100% of the women determined to be infected with a high-oncogenic-risk HPV type progressed to cervical cancer, then type-specific HPV DNA detection would be the “gold standard” of cervical cancer screening. Although more than 99% of cervical cancers contain high-risk HPV, only a small proportion of the women infected with a high oncogenic-risk type will have disease that progresses to cancer. Recent studies also have demonstrated that high-grade lesions also could develop in women infected with non-high-risk types. Whether these non-high-risk lesions that progressed to high-grade lesions developed as a result of increased expression of E6 and E7 mRNA is a distinct possibility if not a necessity. Further prospective studies will be required to confirm this hypothesis.

The Stanford study suggests that the E6 and E7 mRNA assay will be useful in predicting the natural history of patients with HPV

related cervical lesions and could serve as a “secondary marker” for patients who are HPV DNA+. This **will be** most useful in screening young women (less than 30 years old), a cohort in which the prevalence of HPV DNA positivity may be as high as 20% and a population under the age of FDA approval for HC HPV DNA. Furthermore, the E6 and E7 mRNA assay has the potential to function as an adjunct to the Pap smear in screening programs or even as a primary screening tool. HPV E6/E7 also might identify patients with negative Pap

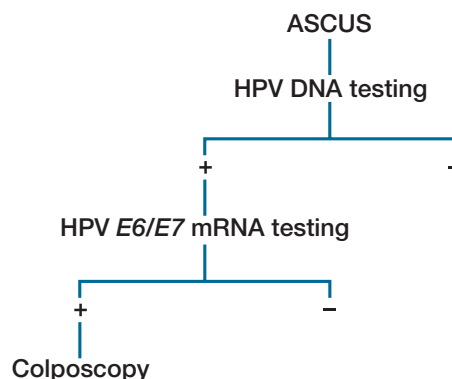
smears in whom the process of cellular transformation into pre-cancerous cells is present.

HPV E6/E7 mRNA screening as an approach to the triaging of ASCUS/HPV DNA positive patients is potentially cost-effective, as the need for colposcopy would be reduced.

Reference:

1. Bosch, F. X., et al. 1995. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J. Natl. Cancer Inst. 87:796-802.
2. ACOG Practice Bulletin. 2005.
3. Solomon, D.; et al. 2001. Comparison of three management strategies for patients with atypical squamous cells of undetermined significance: Baseline results from a randomized trial. J. Nat. Cancer Inst. 2001. 93:293-29
4. Kraus, I., et al. 2006. Presence of E6 and E7E7 mRNA from human papillomavirus types 16, 18, 31, 33, and 45 in the majority of cervical carcinomas. J. Clin. Microbiol. 44(4):1310-1317.
5. Narimatsu, R. and B. Patterson, B. K. 2005. High-throughput cervical cancer screening using intracellular human papillomavirus E6 and E7 mRNA quantification by flow cytometry. Am. J. Pathol. 123:716-723. Please address reprint requests of this published article to: labmarketing@stanfordmed.org

The Potential Role of HPV E6/E7 testing in ASCUS Triaging



Laboratory Update:

New (“Old”) PTH (Parathyroid Hormone) Assay at Stanford Clinical Laboratory

Effective February, 2006, our PTH assay has changed. The test is now performed on Bayer instrumentation. The reference range has changed from 10-60 to 10-80 pg/ml. All reports have a notification regarding this change. Note that levels may be much higher than expected in patients being followed (especially patients with renal insufficiency).

At the Stanford laboratory, we had been using the Nichols Institute Diagnostics assay on their Advantage immunoassay analyzer (called “Bio-Intact PTH”). This so-called “third generation” PTH assay was developed to allow detection of the complete 1-84 hormone. However, late in 2005, the company was no longer able to provide this product. We needed to transfer the PTH assay to another analyzer and chose the Bayer Centaur. The Bayer PTH assay, however, is the old “intact” assay (that “sees” 7-84 PTH as well as the 1-84 intact hormone). See discussion below.

If you have any questions about this change in our PTH assay, please contact Dr. Jim Faix (Director of Clinical Chemistry and Immunology).

PTH DISCUSSION:

Parathyroid hormone (PTH) regulates the extracellular calcium concentration. Parathyroid cells continuously monitor free (or “ionized”) calcium levels and, when they fall, the cells produce PTH which immediately increases tubular reabsorption of calcium. If this action is not sufficient to restore calcium levels to normal, PTH production continues and calcium is mobilized from the bone. PTH also enhances renal conversion of 25-hydroxy vitamin D to 1,25-dihydroxy vitamin D (which promotes calcium absorption from the gut).

PTH action is largely controlled by protease degradation so the hormone has a very short half-life in the blood (approximately 5 minutes). The original (competitive) immunoassays for PTH did not distinguish the intact PTH molecule from the biologically-inactive fragments produced by protease cleavage.

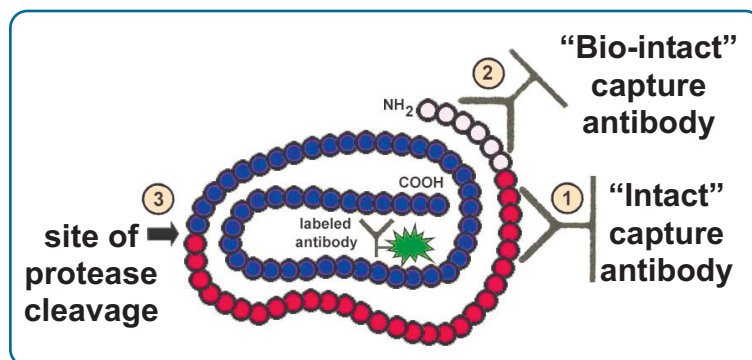
The creation of a non-competitive (“sandwich”) immunoassay, with one antibody directed against the N-terminal portion and another against the C-terminal portion, was a major advance. These so-called “intact” PTH assays were especially helpful for monitoring patients with renal insufficiency because PTH fragments are excreted in the kidney (and therefore elevated in renal failure).

In the late 1990s, investigators discovered a new type of PTH fragment that could be called “almost intact” PTH. This fragment contains residues 7-84 and seems to be a product of the parathyroid cells (not a proteolytic cleavage product). Like the smaller

fragments, these 7-84 versions of PTH do not promote increased calcium levels. (Some researchers believe that they may, in fact, be antagonistic to the action of PTH.) However, it was clear that the existing “intact” PTH immunoassays were able to detect these 7-84 versions of the hormone.

Recently, new (so-called “third generation”) PTH assays were developed to allow detection of only the complete 1-84 hormone. There were two commercially available versions of these: one was a reference test (“Cyclase-Activating PTH” from Scantibodies in Santee, California) and the other was marketed by Nichols Institute Diagnostics for use on their Advantage immunoassay analyzer (called “Bio-Intact PTH”). This latter product is no longer commercially available.

The “cyclase-activating” (or “bio-intact”) PTH assay may be more diagnostically sensitive than the older one for identifying patients with primary hyperparathyroidism and may better identify renal failure patients who would benefit from vitamin D therapy for bone disease. However, both of these claims are controversial.



PTH Image Legend:

PTH immunoassays capture the hormone using an antibody against the N-terminal portion and measure it using a labeled antibody against the C-terminal portion.

1. The N-terminal antibody in older (“intact”) assays actually binds a portion away from the very end of the molecule; this allows them to “see” shorter (7-84) versions of the hormone.
2. Newer (“bio-intact”) assays employ a capture antibody that only detects the complete (1-84) version
3. Neither version detects the fragments produced by proteolytic cleavage.

Q&A: Submit Q&A topics of interest to: labmarketing@stanfordmed.org

An opportunity for the Bay Area Medical Community to request specific Q&A topics to our medical directors that are relevant to patient's clinical needs.



James Faix, M.D.

B-type natriuretic peptide: NT-proBNP vs. BNP Assays

Q&A with Jim Faix, M.D., Director of Clinical Chemistry and Immunology at Stanford Clinical Laboratory and Associate Professor of Pathology at Stanford University School of Medicine.

The topic has been submitted by several physicians (both at Stanford and Outreach) asking why Stanford uses the NT-proBNP assay for B-type natriuretic peptide rather than the BNP assay

Q1: What is BNP?

A1: Natriuretic peptides produced by the heart and blood vessels improve the ability of the left ventricle to pump properly by decreasing peripheral vascular resistance and promoting excretion of sodium and water. These natriuretic peptides appear whenever the left ventricular function is compromised and elevated blood levels are characteristic of congestive heart failure (CHF). Brain-type (or B-type) natriuretic peptide was originally differentiated from atrial natriuretic peptide (ANP) because it was discovered in the brain, but it is primarily produced by the cardiac ventricles. BNP has assumed a prominent role in the management of patients with CHF.

Q2: What is the difference between BNP and NT-proBNP?

A2: BNP is not stored in ventricular myocytes. Hemodynamic stress (and, usually, stretching) activates transcription and the gene is translated within the cell as a 108 amino-acid precursor. On release, this proBNP molecule is cleaved into the 32 amino-acid active hormone (BNP) and an inactive 76 amino-acid peptide. This inactive peptide is called the N-terminal peptide of proBNP (NT-proBNP).

Q3: Why do the two assays produce such different results?

A3: Much has been written about the differences between BNP and NT-proBNP results. Although they appear in the circulation in an equimolar fashion, levels of NT-proBNP are much higher than BNP. Part of this discrepancy is due to the fact that both assays are calibrated in mass units (pg/ml); by definition, NT-proBNP is much larger than BNP (76 amino acids instead of 32). But, even if one accounts for this difference, NT-proBNP levels are still higher. Most of this difference has been attributed to NT-proBNP's longer half-life. BNP, the active hormone, is cleared rapidly because it binds to receptors; it has a half-life of approximately 20 minutes. NT-proBNP's clearance is less well-understood; its half-life is longer (approximately 120 minutes).

Q4: Why does the Stanford laboratory only assay for NT-proBNP?

A4: Although most laboratory investigations have shown the two assays to be equivalent for ruling out and diagnosing CHF, we know that use of NT-proBNP is controversial. We chose to go with NT-proBNP primarily because we are able to assay NT-proBNP on the same instrument that we use for other cardiac markers needed quickly by the Emergency Department (such as troponin). This may change in the future, at which point we might need to switch to BNP. We have considered offering both assays to outreach customers but we would not want this option to be available for patients who may be seen in the Stanford ED (where only NT-proBNP is available) because it might create confusion.

Q5: Why does Stanford use a single cut-off of 300 pg/ml for NT-proBNP?

A5: When Biosite introduced their BNP assay, they recommended a single cut-off for ruling-out CHF: <100 pg/ml. This single cut-off was not chosen because BNP is not affected by age; in fact, it's quite clear that BNP (like NT-proBNP) is higher in patients >75 years of age. Rather, Biosite used receiver-operator curve analysis in a large clinical evaluation of over 1500 patients called the "Breathing Not Properly" (or BNP) study. Recently, the single rule-out cut-off for NT-proBNP has been verified by a similar study called the "Pro-BNP Investigation of Dyspnea in the Emergency Department" (or PRIDE). The single rule-out cut-off found to be optimal in the PRIDE study was <300 pg/ml. This produced a negative predictive value of 99%.

Q6: Are there other clinical applications for NT-proBNP?

A6: There are many other clinical applications of natriuretic peptide testing.

- Levels may help establish a prognosis for patients admitted to hospital with acute CHF.
- Patients may also be followed on an outpatient basis, monitoring the patient's "dry" NT-proBNP (the level that, when correlated with the clinical findings, reassures the physician that the patient is compensated and clinically stable).
- A new area is the management of patients with acute coronary syndrome (ACS). Currently, this is primarily for risk stratification of newly diagnosed cases. But recent reports have shown that certain levels of natriuretic peptide (all well below the cut-off for CHF) or small changes in levels during exercise testing can enhance the initial diagnosis of ACS as well. A better understanding of pre-analytical factors will be important as we move to add these indications.

