

Improving Cervical Screening Through Multiplex Detection and Self-Sampling

Paul Vichi¹, Luisa Escobar-Hoyos^{2,3}, and Kenneth R. Shroyer²

1. OncoGenesis, Inc., Morgan Hill, CA. USA
2. Department of Pathology, Stony Brook Medicine, Stony Brook, New York. USA
3. Human Oncology and Pathogenesis Program (HOPP), Memorial Sloan Kettering Cancer Center, New York, New York. USA

Abstract

Cervical cancer, is the result of infection by sexually transmitted, high risk strains of the human papillomavirus (hrHPV)[1]. Viral persistence and subsequent integration into the human genome induces a number of molecular and cellular changes in cervical epithelial cells, which override normal growth control mechanisms, leading to sustained cell cycle progression and subsequent transformation. Determination of the molecular mechanisms engaged in malignant transformation of the cervical mucosa has led to the discovery of a range of candidate cervical cancer biomarkers that could be used to identify HPV-positive patients at the greatest risk for developing cervical cancer and most likely to benefit from further intervention. Accordingly, evidence continues to accumulate that adjunct testing with single or multiplex cervical cancer biomarkers improves screening; however, these practices have been difficult to deploy in low resource settings, requiring additional approaches such as self sampling, and high throughput analyses to facilitate screening and model validation. Further discovery of the mechanisms that drive the pathogenesis of cervical cancer have the potential to improve adjunct testing, which

could one day supplant normal cytology, and focus the development of novel therapies ultimately improving cervical screening and patient survival in low resource settings where need is greatest.

Cervical Screening – Disease and Guidelines

The American Cancer Society (ACS) screening guidelines for detection of cervical cancer, cosponsored by the American Society for Colposcopy and Cervical Pathology (ASCCP) and the American Society for Clinical Pathology (ASCP), recommend HPV co-testing with cytology every 5 years in women ≥ 30 years of age, and emphasize several priorities for future research[2], among them:

- Strategies that improve screening in unscreened or under-screened women;
- Novel strategies utilizing HPV testing and other molecular approaches, such as self collection of cervico-vaginal specimens for co testing with HPV and cytology;
- Research on the use of novel biomarkers

Detection of precancerous cervical lesions or invasive cancer is typically achieved through a subjective, program of cytological screening using Papanicolaou (PAP) stained cervical specimens. Based upon morphology, cervical specimens are stratified into diagnostic categories, including, normal, atypical squamous cells of undetermined significance (ASC-US), low-grade intraepithelial lesion (LSIL), high-grade intraepithelial lesions (HSIL), and cancer. Although effective in reducing cancer deaths in developed countries, due to the lack of qualified cytopathologists and required infrastructure in many third world settings, it has been challenging

to implement slide-based screening programs for women with the greatest need, forcing clinical care providers to rely on less sensitive and specific methods, such as *in situ* visualization with acetic acid (VIA).

As with many cancers, early detection can improve outcome however, identifying the subset of patients with precancerous lesions that pose the greatest risk can be difficult. This is particularly challenging for women with cytology results that are suspicious (ASC-H) or diagnostic of HSIL, as these are usually flat lesions that may be very difficult to visualize. Similarly, potential premalignant findings of the endocervical glandular mucosa, including atypical glandular cells (AGC) and adenocarcinoma in situ (AIS) may be extremely hard to detect by clinical examination. With potentially greater numbers of women stratified as HPV-positive/cytology negative, or ASC-US, which may harbor underlying HSIL or high-grade endocervical glandular lesions, there is a call to implement methodologies that will improve diagnostic/prognostic accuracy[3].

The challenge in applying differentiating factors as part of cytological testing was evident in an ASCUS-LSIL triage study where patients with LSIL or ASCUS were randomized to 1 of 3 study arms including immediate colposcopy, HPV triage testing using Hybrid Capture 2 or conservative follow-up with cytology only[4]. Although HPV triage proved useful for women with ASCUS, the high incidence of HPV invalidated its use as a triage for women with LSIL. While patients with a cytological diagnosis of ASCUS have a 5% - 17% chance of an underlying

HSIL, the majority of ASCUS and AGC patients were not found to have clinically significant lesions. Patients with LSIL referred for colposcopy, revealed HSIL on biopsy in ~ 25% of cases upon further examination; LSIL in 45% of cases, but no dysplasia in >25%. Furthermore, even patients deemed to have HSIL on cytology have a 24% - 94% chance of high grade cervical intraepithelial neoplasia(CIN2+/3+) upon biopsy[5]. This variability underlies problems of both limited specificity and sensitivity of cervical cytology, reported to range from 1.6 to as much as 28%[5].

Co-Testing – Impact on screening recommendations

The molecular mechanisms underlying HPV-induced cervical transformation support using analyses of additional targets in the diagnosis of high-risk premalignant lesions. Foremost is the inclusion of tests detecting hrHPV strains to provide an additional basis for identifying patients that should be further evaluated with colposcopy/biopsy. Although HPV infection, is a common STD, affecting nearly 79 million Americans with ~ 14 million new cases identified each year[6] [7] most infections clear spontaneously without medical intervention[8]. Thus, the detection of hrHPV has generally poor specificity for underlying premalignant or malignant lesions that require intervention. Although there is evidence that increased HPV copy number is a risk factor for disease progression, the highest levels of HPV are associated with LSIL lesions that harbor productive infections[5] while HPV copy number can be quite low in most HSILs and SCCs [9], precluding its use as a reliable differentiating factor for CIN[10]. Furthermore, the detection of hrHPV DNA or RNA in a cytologic specimen fails to provide information on events such as viral

integration, loss/gain-of-function that drive oncogenic transformation, or that potentially guide intervention.

Current ACS guidelines support HPV and cytology co-testing of women aged 30-65 years of age, every 5 years; however, due to the high rates of infection in younger women, HPV testing is not recommended for women < 30 years of age, nor is it recommended as a standalone assay.

Evidence from this approach demonstrates improved sensitivity for HSIL, reducing patient cancer risk, but a lack of specificity, potentially leads to unnecessary colposcopy/biopsy [11,12].

The limited specificity for high-grade lesions is due in part to the very high rates of transient HPV infections that are cleared by the host's immune system[13]. Although results are encouraging, conclusions on the effectiveness of HPV testing for primary screening will require further evidence, including large-scale, long-term clinical studies. A potential problem with adding HPV detection to cytology is that greater numbers of women may undergo otherwise unnecessary colposcopic examinations, particularly patients that test positive for HPV on repeated screenings, even in the absence of abnormal cytological evidence to support transformation.

It is imperative then to also increase the reassurance provided by negative colposcopy findings by adding metrics that improve colposcopic sensitivity or ideally improve the selection of women forwarded for colposcopy/biopsy. For this group of patients, testing for additional

biomarkers that more closely reflect cellular changes indicative of HPV-induced transformation would provide increased confidence.

Secondary Targets for Triage of HPV (+) Patients

Screening guidelines recognize that secondary testing of cervical cancer biomarkers can improve specificity and disease management, however, the limited data regarding test performance has hindered adoption. Until such time that additional biomarkers can be clinically validated as both safe and effective adjuncts or alternatives to HPV testing, the ACS has recommended continued research into the use of novel biomarkers to improve the management of cervical cancer screening/diagnosis, particularly in women with HPV-positive/cytology-negative co-test results[14].

Examination of mRNA species that may be altered in CIN has been proposed as a tool to provide greater of cellular changes underlying transformation, however, studies examining viral mRNA levels in CIN were inconsistent and not strongly supportive of using HPV mRNA as a marker to gauge malignant transformation[15,16]. Recent evidence suggests that microRNA expression in HPV-induced cervical lesions could provide alternative biomarkers for evaluation[17] however, as with the analysis of mRNA, qPCR processes for microRNA are demanding. Without unique implementation approaches, the expense, time and technical requirements do not favor their adoption in low resources settings, yet they remain powerful tools for collecting data necessary for accurately modeling HPV-induced transformation.

Expression of both host and viral genes is influenced by CpG methylation patterns and several candidate host genes, which function as negative regulators of cell growth and motility, display altered methylation frequency, which correlates with increase severity of cervical dysplasia[18]. Looking beyond the presence of nucleic acids indicative of high-risk HPV, the methylation status of specific genes may serve as an effective co-test for triage in HPV positive patients, demonstrating improvements in specificity with similar sensitivity as combined Pap smear/HPV testing[19–21]. Methylation status can also be readily identified with next generation sequencing (NGS) instruments used for detecting specific HPV strains[22].

Insight gained from understanding the life cycle of HPV and the interaction of viral oncoproteins with a variety of host cell factors[23,24] supports using multiple potential biomarkers to triage HPV-positive/cytology-negative or ASC-US patients including proteins important in the cell cycle[25–27], cell signaling[28], apoptosis[29,30], and epigenetic modification of the host genome[31–33]. Persistent infection with hrHPV can lead to integration of HPV DNA within the host genome, which often precedes transformation[34]. In productive episomal infections, the expression of early viral genes E1 and E2 suppress HPV E6 and E7 gene expression. Viral integration however, disrupts E1 and E2, enabling the overexpression of E6 and E7 viral oncoproteins, which target cell cycle regulatory proteins, including p53[35–37], Rb[38,39], and MAPK[40] in cervical cells. Alternative splicing of the E6 transcripts (E6*), observed only in hrHPV, was further associated with changes in β -integrin signaling, mitochondrial dysfunction

pathways, oxidative phosphorylation[41], and EGF[42] signaling, providing not only additional protein targets but suggesting the importance of other nucleic-acid-based targets whose inclusion may increase specificity, or predictive value in HPV-based screening approaches.

Candidate protein biomarkers as adjunct tools to triage HPV-positive patients include p16^{ink4a}, minichromosome maintenance (MCM) family proteins, topoisomerase, E6, survivin, CDC6, telomerase and keratin-17 (K17)[43–49]. Among these, evidence of an adjunct role for p16^{ink4a} is strongest where overexpression follows E7-induced Rb degradation, demonstrating a close correlation with CIN grade[50]. Studies also support detection of CDC6, MCMs and topoisomerase 2 α as proteins of aberrant cell proliferation underlying but not necessarily specific of high-grade dysplasia[26,43,51–53]. Conversely, though the full mechanism of how K17 is upregulated during cervical cell transformation remains unclear, it appears independent of HPV oncoprotein expression[27] contributing to transformation by targeting and promoting the degradation of p27, a crucial tumor cell cycle-related tumor suppressor and by potentially polarizing the immune system[54]. K17 expression is now identified as a novel diagnostic biomarker specific and sensitive for the detection of HSILs and SCCs[27,55] revealing the most aggressive and lethal forms of cervical cancers.

Inhibition of apoptosis is another fundamental hallmark of many cancers and is regulated by both Bcl-2 and inhibitor of apoptosis protein (IAP) family members. Survivin is unique among the human IAPs because it appears to be expressed in most cancers but is rarely detected in

corresponding normal adult somatic tissues. As first reported in 2002, the nuclear expression of survivin is correlated with morphologic and molecular evidence of human papillomavirus (HPV) infection in premalignant and malignant lesions of the cervical mucosa.[46] Further studies have confirmed that survivin might be developed as a biomarker to increase diagnostic accuracy for cervical screening[30,56–59].

The potential of protein biomarkers has led to the introduction of several commercial kits including Roche/Ventana's CINtec® p16 Histology and CINtec PLUS Cytology® kits, approved for use in Europe; the ProExC kit produced by Becton Dickinson (BD) for cytological analysis of topoisomerase II and MCM proteins using immunocytochemical or immunohistochemical approaches[52]; and Arbor Vita's OncoE6™ lateral flow assay for cervical testing of E6 oncoprotein, approved for use in Europe and compatible with testing on Pap smear and liquid cervical specimens. Most of these kits, however, rely on IHC of cytology or biopsy samples limiting use in underserved region. In other cases, studies based on single marker analysis have been inconsistent [60,61], which may also limit their use as adjunct tests. Specificity and sensitivity however, can be improved by using a multiplexed approach for assay of cellular factors whose level or activity is altered following HPV integration. For example, combining analysis of p16 and Ki-67 in cytology specimens provided significant improvement in specificity and sensitivity for the diagnosis of CIN2/3 or glandular lesions compared to PCR-based HPV testing[62–65]. The CINTEC® Plus kit, which examines both p16^{ink4a} and Ki-67 using a double staining approach on cytology specimens also showed improved detection of high-grade lesions [64].

Similarly, immunohistochemical detection of 13 biomarkers in >300 cervical biopsy samples demonstrated the power of multiplex detection to improve sensitivity and specificity in the grading of CIN[66]. Table 1, created from the same data illustrates the added benefit for biopsy testing and in a hypothetical screening series, in improved sensitivity, NPV, and ROCs when using multiplex analysis of p16^{ink4a} and survivin. ROC values using multiplex detection are improved relative to single marker sampling, but as with the cytological staining kits described above, IHC staining practices can be very demanding and thus have limited application for rapid triage or to collect the massive amounts of data needed for model assembly.

Table 1: p16 and Survivin Biopsy Data (extracted from Branca et al., Inter J Gynecol Pathol. 2008; 27:265-273; personal communication S. Syrjanen).

| Marker | Sensitivity % (95%CI) | Specificity % (95%CI) | PPV % (95%CI) | NPV % (95%CI) | Area under ROC curve | OR (95%CI) |
|--|-------------------------|-------------------------|-------------------------|-------------------------|----------------------------|--------------------------|
| BIOPSY SERIES* | | | | | | |
| p16 ^{INK4a} | 54.4 (48.1-60.5) | 93.1 (77.2-99.2) | 98.6 (95.1-99.8) | 18.4 (12.5-25.6) | 0.737 (0.682-0.793) | 16.08 (3.74-69.04) |
| Survivin | 82.5 (77.3-86.9) | 75.9 (56.5-89.7) | 96.8 (93.5-98.7) | 32.8 (21.8-45.4) | 0.792 (0.709-0.874) | 14.80 (5.96-36.75) |
| p16 + Survivin | 85.7 (80.9-86.6) | 73.3 (54.1-87.7) | 96.7 (93.6-98.6) | 36.1 (24.2-49.5) | 0.796 (0.712-0.878) | 16.4 (6.94-38.8) |
| SCREENING SETTING (N=10,000): CIN2+ PREVALENCE 1%, TEST SE AND FALSE POSITIVE RATE KEPT AS IN BIOPSY SERIES | | | | | | |
| p16 ^{INK4a} | 54.4 (44.7-65.0) | 99.3 (99.1-99.5) | 44.7 (35.7-53.9) | 99.5 (99.4-99.7) | 0.772 (0.723-0.821) | 179.0 (113.0-282.0) |
| Survivin | 82.5 (74.2-89.8) | 97.6 (97.2-97.9) | 25.4 (20.8-30.5) | 99.8 (99.7-99.9) | 0.903 (0.866-0.940) | 195 (115.0-332.0) |
| p16 + Survivin | 85.7 (77.6-92.1) | 97.4 (97.1-97.7) | 24.9 (20.4-29.8) | 99.9 (99.8-99.9) | 0.917 (0.883-0.951) | 230 (130.0-407.0) |

*Biopsy Series (n=302): CIN1(LSIL) (n=30** includes 10 cases of NCIN); CIN2 (n=21); CIN3 (n=101); SCC (n=150); FPR=false positive rate (FP/N)

Determining threshold values for multiple biomarkers, which reflect transformation or provide prognostic value requires examination of large numbers of patient specimens and implementation of quantitative, high throughput analyses, not readily available in low resource settings. These areas currently offer minimal cervical screening and when present, often rely on VIA, which is

less sensitive than cytology[67]. Protein or nucleic acid biomarkers for cervical cancer could be screened using cost effective high throughput processes, and threshold values for multiple viral and host cell factors, which correlate with high risk lesions determined. In this manner, cytology screening may eventually be supplanted by quantitative approach examining a group of viral and host cell factors.

Bridges to New Paradigms

Adoption of new screening metrics in low resource settings will be limited by existing infrastructure and regional capabilities, but may be advanced by combining access to high-throughput technology with self-sampling tools. In the same way that visual inspection with acetic acid can be aided by telemedicine using digital images captured with a smartphone[68], cervical screening sensitivity and specificity could be improved by multiplex genomic or protein-based screening conducted on self-collected cervical specimens sent to a remote facility. This approach provides an intermediate solution to bring high-throughput technology to the analysis of cervical specimens from developing regions, without having to wait for local deployment of costly infrastructure. Although initial reports indicated that self-collected vaginal samples displayed reduced sensitivity and specificity[69], recent data using a NGS-based MALDI-TOF MS genotyping assay assay[70] showed self-collected samples have similar sensitivity for detection of HPV and \geq CIN 3 as an endocervical specimen collected by a trained physician[71]. Self-sampling can also be used to evaluate other metrics including methylation or protein status, demonstrating a close correlation with physician-collected cervical specimens[72,73] making

this an attractive approach to bring alternative screening and improved technology to patients in underserved regions.

The advantage of a self-sampling is being promoted in developed countries currently using cytology, but which recognize that a significant portion of cervical cancers arise in women who elect not to participate in clinical-based testing. In 2016 the Netherlands will be the first European country to switch the basis for cervical screening for women ages 30-60 from cytology to HPV-based detection, using cytology as a co-screen for HPV-positive patients, and providing a self-sampling kit for hrHPV testing to women who fail to respond to invitations for clinical evaluations[74]. Combined with collection tools for improved endocervical self-sampling, which minimize the contamination of factors originating from surrounding tissues, important for establishing threshold values for individual markers, remote options for multiplex detection of biomarker proteins and/or nucleic acids from the same self-collected specimen can be implemented, bridging technology and screening in low resources areas.

Costs Associated with Different Approaches to Cervical Screening

A major impediment to the establishment of screening services in low resource settings is the cost related to infrastructure and testing, which varies depending upon the approach. A micro-costing study comparing the medical expenses of screening and diagnosis in rural China using VIA, combined VIA/VILI, HPV sampling, colposcopy, biopsy, and ECC, showed that aggregate costs of HPV testing average more than 3 times the expense of VIA or combined VIA/VILI

testing, with laboratory costs and HPV kits comprising the majority of the expense[75]. Adding on direct medical costs of colposcopy, biopsy and or ECC for HPV-positive women rapidly expands the cost of cervical screening and diagnosis. The China study also illustrated the importance of high-volume screening to maintain lower costs, arguing for centralized approaches. The expense of screening/diagnostics for 2000 people a year in China, were roughly three times the cost of examining 6000 or more patients, highlighting the need of high-throughput platforms to achieve cost efficiency. A cost-effective analysis examining the health outcomes and expenses associated with Pap, HPV or combination-based screening in Mexico demonstrated that co-testing with HPV and Pap screening improves health benefits at reasonable costs compared with current approaches[76]. These data were limited however, in that the true cost of missed diagnosis is difficult to assess. Using published estimates (2007) of \$1610 to treat CIN2/3 or \$8421 to treat cervical cancer in Mexico [77], significant savings could be captured by improving early detection through added triage metrics. Using HPV as a sole metric; however, or in combination with ASC-US will result in far high numbers of women being subjected to unnecessary colposcopy or biopsy, increasing costs.

Conclusions

Tests for multiplex biomarker detection that accurately grade cervical cancer risk in HPV-positive specimens would provide significant savings while ensuring only those women with hrHPV and evidence of transformation are forwarded for diagnostic confirmation and treatment. As NGS and other high-throughput approaches mature, the cost of nucleic acid or protein-based

detection should decline facilitating application to low resource settings where need is greatest. Combining self-sampling with quantitative assays for cervical biomarkers that can be determined at a central lab or eventually the point-of-care, would provide even further cost reductions facilitating the collection of data necessary for validating models and ensuring prognostic value of biomarkers while improving the delivery of cervical screening to underserved regions.

Acknowledgements:

The authors would like to thank Drs. K. Syrjanen and N. Kim for data discussion.

Funding: None

Competing interests: None declared

Ethical approval: Not required

- [1] J.M.M. Walboomers, M. V. Jacobs, M.M. Manos, F.X. Bosch, J.A. Kummer, K. V. Shah, P.J.F. Snijders, J. Peto, C.J.L.M. Meijer, N. Muñoz, Human papillomavirus is a necessary cause of invasive cervical cancer worldwide, *J. Pathol.* 189 (1999) 12–19. doi:10.1002/(SICI)1096-9896(199909)189:1<12::AID-PATH431>3.0.CO;2-F.
- [2] D. Saslow, D. Solomon, American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the Prevention and Early Detection of Cervical Cancer, *Cancer J. Clin.* 62 (2012) 147–72.

doi:10.3322/caac.21139.American.

- [3] M. Schiffman, N. Wentzensen, A Suggested Approach to Simplify and Improve Cervical Screening in the United States., *J. Low. Genit. Tract Dis.* 20 (2016) 1–7. doi:10.1097/LGT.000000000000170.
- [4] M. Schiffman, D. Solomon, Findings to date from the ASCUS-LSIL Triage Study (ALTS), in: *Arch. Pathol. Lab. Med.*, 2003: pp. 946–949. doi: 10.1043/1543-2165(2003)127<946:FTDFTA>2.0.CO;2.
- [5] D. Dehn, K.C. Torkko, K.R. Shroyer, Human papillomavirus testing and molecular markers of cervical dysplasia and carcinoma, *Cancer.* 111 (2007) 1–14. doi:10.1002/cncr.22425.
- [6] C.L. Satterwhite, E. Torrone, E. Meites, E.F. Dunne, R. Mahajan, M.C.B. Ocfemia, J. Su, F. Xu, H. Weinstock, Sexually transmitted infections among US women and men: prevalence and incidence estimates, 2008., *Sex. Transm. Dis.* 40 (2013) 187–93. doi: 10.1097/OLQ.0b013e318286bb53.
- [7] Centers for Disease Control, Human papillomavirus-associated cancers - United States, 2004-2008., *MMWR. Morb. Mortal. Wkly. Rep.* 61 (2012) 258–61. <http://www.ncbi.nlm.nih.gov/pubmed/22513527>.
- [8] E.L. Franco, L.L. Villa, J.P. Sobrinho, J.M. Prado, M.C. Rousseau, M. Desy, T.E. Rohan, Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high-risk area for cervical cancer, *J. Infect. Dis.* 180 (1999) 1415–1423. doi:JID990342 [pii]\r10.1086/315086.

- [9] J. Doorbar, Molecular biology of human papillomavirus infection and cervical cancer, *Clin Sci.* 110 (2006) 525–541. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16597322.
- [10] G.A. V Boulet, I.H. Benoy, C.E. Depuydt, C.A.J. Horvath, M. Aerts, N. Hens, A.J. Vereecken, J.J. Bogers, Human papillomavirus 16 load and E2/E6 ratio in HPV16-positive women: Biomarkers for cervical intraepithelial neoplasia ≥ 2 in a liquid-based cytology setting?, *Cancer Epidemiol. Biomarkers Prev.* 18 (2009) 2992–2999. doi: 10.1158/1055-9965.EPI-09-0025.
- [11] G. Ronco, P. Giorgi-Rossi, F. Carozzi, M. Confortini, P. Dalla Palma, A. Del Mistro, B. Ghiringhello, S. Girlando, A. Gillio-Tos, L. De Marco, C. Naldoni, P. Pierotti, R. Rizzolo, P. Schincaglia, M. Zorzi, M. Zappa, N. Segnan, J. Cuzick, N.T. for C.C. screening (NTCC) W. Group, Efficacy of human papillomavirus testing for the detection of invasive cervical cancers and cervical intraepithelial neoplasia: a randomised controlled trial., *Lancet Oncol.* 11 (2010) 249–257. doi:S1470-2045(09)70360-2 [pii] 10.1016/S1470-2045(09)70360-2.
- [12] S. Kjaer, E. Høgdall, K. Frederiksen, C. Munk, A. Van Den Brule, E. Svare, C. Meijer, A. Lorincz, T. Iftner, The absolute risk of cervical abnormalities in high-risk human papillomavirus-positive, cytologically normal women over a 10-year period, *Cancer Res.* 66 (2006) 10630–10636. doi:10.1158/0008-5472.CAN-06-1057.
- [13] M. Schiffman, N. Wentzensen, Human papillomavirus infection and the multistage carcinogenesis of cervical cancer, *Cancer Epidemiol. Biomarkers Prev.* 22 (2013) 553–

560. doi:10.1158/1055-9965.EPI-12-1406.
- [14] D. Saslow, D. Solomon, H.W. Lawson, M. Killackey, S.L. Kulasingam, J. Cain, F.A.R. Garcia, A.T. Moriarty, A.G. Waxman, D.C. Wilbur, N. Wentzensen, L.S. Downs, M. Spitzer, A.-B. Moscicki, E.L. Franco, M.H. Stoler, M. Schiffman, P.E. Castle, E.R. Myers, American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer., *CA. Cancer J. Clin.* 62 (2012) 147–72. doi:10.3322/caac.21139.
- [15] M. Persson, S.B. Wendel, L. Ljungblad, B. Johansson, E. Weiderpass, S. Andersson, High-risk human papillomavirus E6/E7 mRNA and L1 DNA as markers of residual/recurrent cervical intraepithelial neoplasia, *Oncol. Rep.* 28 (2012) 346–352. doi:10.3892/or.2012.1755.
- [16] P. Value, H. Papillomavirus, M.G. Discacciati, I. Dcg, L.L. Villa, L. Reis, P. Hayashi, M.C. Costa, S.H. Rabelo-santos, L.C. Zeferino, *Biomarker Insights*, (2014) 15–22. doi:10.4137/BMI.S14296.Received.
- [17] K. Gocze, K. Gombos, K. Kovacs, K. Juhasz, P. Gocze, I. Kiss, MicroRNA expressions in HPV-induced cervical dysplasia and cancer, *Anticancer Res.* 35 (2015) 523–530. doi:35/1/523 [pii].
- [18] M. a Clarke, N. Wentzensen, L. Mirabello, A. Ghosh, S. Wacholder, A. Harari, A. Lorincz, M. Schiffman, R.D. Burk, Human papillomavirus DNA methylation as a potential biomarker for cervical cancer., *Cancer Epidemiol. Biomarkers Prev.* 21 (2012) 2125–37.

doi:10.1158/1055-9965.EPI-12-0905.

- [19] H.C. Lai, Y.C. Ou, T.C. Chen, H.J. Huang, Y.M. Cheng, C.H. Chen, T.Y. Chu, S.T. Hsu, C. Bin Liu, Y.C. Hung, K.C. Wen, M.H. Yu, K.L. Wang, PAX1/SOX1??DNA methylation and cervical neoplasia detection: A Taiwanese Gynecologic Oncology Group (TGOG) study, *Cancer Med.* 3 (2014) 1062–1074. doi:10.1002/cam4.253.
- [20] Z.M. Wang, PAX1 methylation analysis by MS-HRM is useful in triage of high-grade squamous intraepithelial lesions, *Asian Pacific J. Cancer Prev.* 15 (2014) 891–894. doi:10.7314/APJCP.2014.15.2.891.
- [21] Y.-L. Liou, Y. Zhang, Y. Liu, L. Cao, C.-Z. Qin, T.-L. Zhang, C.-F. Chang, H.-J. Wang, S.-Y. Lin, T.-Y. Chu, Y. Zhang, H.-H. Zhou, Comparison of HPV genotyping and methylated ZNF582 as triage for women with equivocal liquid-based cytology results., *Clin. Epigenetics.* 7 (2015) 50. doi:10.1186/s13148-015-0084-2.
- [22] D.R. Masser, D.R. Stanford, W.M. Freeman, Targeted DNA methylation analysis by next-generation sequencing., *J. Vis. Exp.* (2015) 1–11. doi:10.3791/52488.
- [23] E.-K. Yim, J.-S. Park, Biomarkers in cervical cancer., *Biomark. Insights.* 1 (2007) 215–225.
- [24] E.-K. Yim, J.-S. Park, The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis., *Cancer Res. Treat.* 37 (2005) 319–24. doi:10.4143/crt.2005.37.6.319.
- [25] I. Lesnikova, M. Lidang, S. Hamilton-Dutoit, J. Koch, p16 as a diagnostic marker of cervical neoplasia: a tissue microarray study of 796 archival specimens., *Diagn. Pathol.* 4

- (2009) 22. doi:10.1186/1746-1596-4-22.
- [26] N. Murphy, M. Ring, a G. Killalea, V. Uhlmann, M. O'Donovan, F. Mulcahy, M. Turner, E. McGuinness, M. Griffin, C. Martin, O. Sheils, J.J. O'Leary, p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears., *J. Clin. Pathol.* 56 (2003) 56–63. doi:10.1136/jcp.56.1.56.
- [27] L.F. Escobar-Hoyos, R. Shah, L. Roa-Peña, E.A. Vanner, N. Najafian, A. Banach, E. Nielsen, R. Al-Khalil, A. Akalin, D. Talmage, K.R. Shroyer, Keratin-17 promotes p27KIP1 nuclear export and degradation and offers potential prognostic utility, *Cancer Res.* 75 (2015) 3650–3662. doi:10.1158/0008-5472.CAN-15-0293.
- [28] M. Branca, M. Ciotti, D. Santini, L. Di Bonito, A. Benedetto, C. Giorgi, P. Paba, C. Favalli, S. Costa, A. Agarossi, M. Alderisio, K. Syrj?nen, Activation of the ERK/MAP kinase pathway in cervical intraepithelial neoplasia is related to grade of the lesion but not to high-risk human papillomavirus, virus clearance, or prognosis in cervical cancer, *Am. J. Clin. Pathol.* 122 (2004) 902–911. doi:10.1309/VQXF-T880-JXC7-QD2W.
- [29] M. Branca, C. Giorgi, D. Santini, L. Di Bonito, M. Ciotti, S. Costa, A. Benedetto, E.A. Casolati, C. Favalli, P. Paba, P. Di Bonito, L. Mariani, S. Syrj?nen, D. Bonifacio, L. Accardi, F. Zanconati, K. Syrj?nen, Survivin as a marker of cervical intraepithelial neoplasia and high-risk human papillomavirus and a predictor of virus clearance and prognosis in cervical cancer, *Am. J. Clin. Pathol.* 124 (2005) 113–121. doi:10.1309/L8BW-F431-WU9A-C8FJ.
- [30] A. a Borb?ly, M. Murvai, J. K?nya, Z. Beck, L. Gergely, F. Li, G. Veress, Effects of

- human papillomavirus type 16 oncoproteins on survivin gene expression., *J. Gen. Virol.* 87 (2006) 287–94. doi:10.1099/vir.0.81067-0.
- [31] Y.-Y. Kan, Y.-L. Liou, H.-J. Wang, C.-Y. Chen, L.-C. Sung, C.-F. Chang, C.-I. Liao, PAX1 Methylation as a Potential Biomarker for Cervical Cancer Screening., *Int. J. Gynecol. Cancer.* 24 (2014) 928–34. doi:10.1097/IGC.000000000000155.
- [32] M.K. Kim, I.H. Lee, K.H. Lee, Y.K. Lee, K.A. So, S.R. Hong, C.S. Hwang, M.K. Kee, J.E. Rhee, C. Kang, S.Y. Hur, J.S. Park, T.J. Kim, DNA methylation in human papillomavirus-infected cervical cells is elevated in high-grade squamous intraepithelial lesions and cancer, *J. Gynecol. Oncol.* 27 (2016). doi:10.3802/jgo.2016.27.e14.
- [33] M.E. McLaughlin-Drubin, K. Munger, Biochemical and functional interactions of human papillomavirus proteins with polycomb group proteins, *Viruses.* 5 (2013) 1231–1249. doi:10.3390/v5051231.
- [34] J.G. Baseman, L.A. Koutsky, The epidemiology of human papillomavirus infections, *J. Clin. Virol.* 32 (2005). doi:10.1016/j.jcv.2004.12.008.
- [35] F. Hoppe-Seyler, K. Butz, Repression of endogenous p53 transactivation function in HeLa cervical carcinoma cells by human papillomavirus type 16 E6, human mdm-2, and mutant p53., *J. Virol.* 67 (1993) 3111–3117.
- [36] M. Thomas, D. Pim, L. Banks, The role of the E6-p53 interaction in the molecular pathogenesis of HPV, *Oncogene.* 18 (1999) 7690–7700. doi:10.1038/sj.onc.1202953.
- [37] D. Martinez-Zapien, F.X. Ruiz, J. Poirson, A. Mitschler, J. Ramirez-Ramos, A. Forster, Structure of the E6/E6AP/p53 complex required for HPV-mediated degradation of p53,

- Nature. 529 (2016) 541–545.
- [38] S.N. Boyer, D.E. Wazer, V. Band, E7 Protein of Human Papilloma Virus-16 Induces Degradation of Retinoblastoma Protein through the Ubiquitin-Proteasome Pathway
Advances in Brief E7 Protein of Human Papilloma Virus-16 Induces Degradation of Retinoblastoma Protein through the Ubiquitin-Prot, (1996) 4620–4624.
- [39] A. a Bahnassy, A.R.N. Zekri, M. Saleh, M. Lotayef, M. Moneir, O. Shawki, The possible role of cell cycle regulators in multistep process of HPV-associated cervical carcinoma., BMC Clin. Pathol. 7 (2007) 4. doi:10.1186/1472-6890-7-4.
- [40] O. Chakrabarti, K. Veeraraghavalu, Y. Liu, E.J. Androphy, A. Margaret, S. Krishna, V. Tergaonkar, M.A. Stanley, Human Papillomavirus Type 16 E6 Amino Acid 83 Variants Enhance E6-Mediated MAPK Signaling and Differentially Regulate Tumorigenesis by Notch Signaling and Oncogenic Ras Human Papillomavirus Type 16 E6 Amino Acid 83 Variants Enhance E6-Mediated MAPK Signal, J. Virol. 78 (2004) 5934–5945. doi: 10.1128/JVI.78.11.5934.
- [41] W. Evans, M. Filippova, V. Filippov, S. Bashkirova, G. Zhang, M.E. Reeves, P. Duerksen-hughes, Overexpression of HPV16 E6 * Alters β -Integrin and Mitochondrial Dysfunction Pathways in Cervical Cancer Cells, Cancer Genomics Proteomics. 13 (2016) 259–273.
- [42] S. Rosenberger, J. De-Castro Arce, L. Langbein, R.D.M. Steenbergen, F. Rösl, Alternative splicing of human papillomavirus type-16 E6/E6* early mRNA is coupled to EGF signaling via Erk1/2 activation., Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 7006–11. doi: 10.1073/pnas.1002620107.

- [43] G.H. Williams, P. Romanowski, L. Morris, M. Madine, A.D. Mills, K. Stoeber, J. Marr, R.A. Laskey, N. Coleman, Improved cervical smear assessment using antibodies against proteins that regulate DNA replication., *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 14932–7. doi:10.1073/pnas.95.25.14932.
- [44] A.S. Rocha, M.C. Bozzetti, L.S. Kirschnick, M.I.A. Edelweiss, Antibody anti-p16INK4a in cervical cytology, *Acta Cytol.* 53 (2009) 253–262. http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L354954261\nhttp://www.acta-cytol.com/toc/auto_article_process.php?year=2009&page=253.
- [45] J. Ling, D. Ph, U. Wiederkehr, S. Cabiness, K.R. Shroyer, J.P. Robinson, Application of Flow Cytometry for Biomarker-Based Cervical Cancer Cells Detection, *Diagn. Cytopathol.* 36 (2008) 76–84. doi:10.1002/dc.
- [46] M. Frost, E.A. Jarboe, D. Orlicky, R. Gianani, L.C. Thompson, T. Enomoto, K.R. Shroyer, Immunohistochemical localization of survivin in benign cervical mucosa, cervical dysplasia, and invasive squamous cell carcinoma, *Am. J. Clin. Pathol.* 117 (2002) 738–744. doi:10.1309/6V09-38K3-JQ40-UR50.
- [47] L. Bonds, P. Baker, C. Gup, K.R. Shroyer, Immunohistochemical localization of Cdc6 in squamous and glandular neoplasia of the uterine cervix, *Arch. Pathol. Lab. Med.* 126 (2002) 1163–1168.
- [48] E.A. Jarboe, K.L. Liaw, L.C. Thompson, D.E. Heinz, P.L. Baker, J.A. McGregor, T. Dunn, J.E. Woods, K.R. Shroyer, Analysis of telomerase as a diagnostic biomarker of cervical dysplasia and carcinoma, *Oncogene.* 21 (2002) 664–673. doi:10.1038/sj.onc.1205073.

- [49] S.J. Hwang, K.R. Shroyer, Biomarkers of cervical dysplasia and carcinoma, *J. Oncol.* (2012). doi:10.1155/2012/507286.
- [50] P. Samarawardana, D.L. Dehn, M. Singh, D. Franquemont, C. Thompson, L. Gaido, K.C. Torkko, P. Homer, S. Burke, M.A. Titmus, V. Nayi, K.R. Shroyer, P16INK4a is superior to high-risk human papillomavirus testing in cervical cytology for the prediction of underlying high-grade dysplasia, *Cancer Cytopathol.* 118 (2010) 146–156. doi:10.1002/cncy.20078.
- [51] A. Freeman, L.S. Morris, A.D. Mills, K. Stoeber, R.A. Laskey, G.H. Williams, N. Coleman, Minichromosome maintenance proteins as biological markers of dysplasia and malignancy, *Clin. Cancer Res.* 5 (1999) 2121–2132.
- [52] K.R. Shroyer, P. Homer, D. Heinz, M. Singh, Validation of a novel immunocytochemical assay for topoisomerase II- α and minichromosome maintenance protein 2 expression in cervical cytology, *Cancer.* 108 (2006) 324–330. doi:10.1002/cncr.22171.
- [53] N. Murphy, M. Ring, C.C.B.B. Heffron, B. King, A.G. Killalea, C. Hughes, C.M. Martin, E. McGuinness, O. Sheils, J.J. O’Leary, p16INK4A, CDC6, and MCM5: predictive biomarkers in cervical preinvasive neoplasia and cervical cancer., *J. Clin. Pathol.* 58 (2005) 525–34. doi:10.1136/jcp.2004.018895.
- [54] R.P. Hobbs, A.S. Batazzi, M.C. Han, P.A. Coulombe, Loss of Keratin 17 induces tissue-specific cytokine polarization and cellular differentiation in HPV16-driven cervical tumorigenesis in vivo., *Oncogene.* (2016). doi:10.1038/onc.2016.102.
- [55] L.F. Escobar-Hoyos, J. Yang, J. Zhu, J.-A. Cavallo, H. Zhai, S. Burke, A. Koller, E.I.

- Chen, K.R. Shroyer, Keratin 17 in premalignant and malignant squamous lesions of the cervix: proteomic discovery and immunohistochemical validation as a diagnostic and prognostic biomarker., *Mod. Pathol. an Off. J. United States Can. Acad. Pathol. Inc.* 27 (2014) 621–630. doi:10.1038/modpathol.2013.166.
- [56] M. Branca, C. Giorgi, D. Santini, L. Di Bonito, M. Ciotti, S. Costa, A. Benedetto, E.A. Casolati, C. Favalli, P. Paba, P. Di Bonito, L. Mariani, S. Syrjänen, D. Bonifacio, L. Accardi, F. Zanconati, K. Syrjänen, Survivin as a marker of cervical intraepithelial neoplasia and high-risk human papillomavirus and a predictor of virus clearance and prognosis in cervical cancer, *Am. J. Clin. Pathol.* 124 (2005) 113–121. doi:10.1309/L8BW-F431-WU9A-C8FJ.
- [57] J.P. Lee, K.H. Chang, J.H. Han, H.S. Ryu, Survivin, a novel anti-apoptosis inhibitor, expression in uterine cervical cancer and relationship with prognostic factors, *Int. J. Gynecol. Cancer.* 15 (2005) 113–119. doi:10.1111/j.1048-891X.2005.15011.x.
- [58] H.-Q. Liu, Y.-H. Wang, L.-L. Wang, M. Hao, P16INK4A and survivin: Diagnostic and prognostic markers in cervical intraepithelial neoplasia and cervical squamous cell carcinoma., *Exp. Mol. Pathol.* 99 (2015) 44–9. doi:10.1016/j.yexmp.2015.04.004.
- [59] X.L. Zhou, M. Wang, Expression levels of survivin, Bcl-2, and KAI1 proteins in cervical cancer and their correlation with metastasis, *Genet. Mol. Res.* 14 (2015) 17059–17067.
- [60] M. Benevolo, A. Vocaturo, M. Mottolese, L. Mariani, G. Vocaturo, F. Marandino, I. Sperduti, F. Rollo, B. Antoniani, R.P. Donnorso, Clinical role of p16INK4a expression in liquid-based cervical cytology : Correlation with HPV testing and histologic diagnosis,

- Am. J. Clin. Pathol. 129 (2008) 606–612. doi:10.1309/BEPQXTCQD61RGFMJ.
- [61] N.J. Mulvany, D.G. Allen, S.M. Wilson, Diagnostic utility of p16INK4a: a reappraisal of its use in cervical biopsies., *Pathology*. 40 (2008) 335–344. doi: 10.1080/00313020802035907.
- [62] M. Singh, D. Mockler, A. Akalin, S. Burke, A.L. Shroyer, K.R. Shroyer, Immunocytochemical colocalization of P16INK4a and Ki-67 predicts CIN2/3 and AIS/ adenocarcinoma pilot studies, *Cancer Cytopathol*. 120 (2012) 26–34. doi:10.1002/cncy. 20188.
- [63] P. Samarawardana, M. Singh, K.R. Shroyer, Dual stain immunohistochemical localization of p16INK4A and ki-67: a synergistic approach to identify clinically significant cervical mucosal lesions., *Appl Immunohistochem Mol Morphol*. 19 (2011) 514–518. doi:10.1097/ PAI.0b013e3182167c66.
- [64] T. Yoshida, T. Sano, T. Kanuma, H. Inoue, T. Itoh, C. Yazaki, M. Obara, T. Fukada, Usefulness of CINtec PLUS p16INK4a/Ki-67 double-staining in cytological screening of cervical cancer, *Acta Cytol*. 55 (2011) 413–420.
- [65] N. Wentzensen, M. Schiffman, T. Palmer, M. Arbyn, Triage of HPV positive women in cervical cancer screening, *J. Clin. Virol*. 76 (2015) S49–S55. doi:10.1016/j.jcv. 2015.11.015.
- [66] M. Branca, M. Ciotti, C. Giorgi, D. Santini, L. Di Bonito, S. Costa, A. Benedetto, D. Bonifacio, P. Di Bonito, P. Paba, L. Accardi, S. Syrjänen, C. Favalli, K. Syrjänen, Predicting high-risk human papillomavirus infection, progression of cervical

- intraepithelial neoplasia, and prognosis of cervical cancer with a panel of 13 biomarkers tested in multivariate modeling., *Int. J. Gynecol. Pathol.* 27 (2008) 265–273. doi:10.1097/PGP.0b013e318159cbc0.
- [67] J. Bigoni, M. Gundar, P.-M. Tebeu, A. Bongoe, S. Schäfer, J. Fokom-Domgue, R. Catarino, E. Foguem Tincho, S. Bougel, P. Vassilakos, P. Petignat, Cervical cancer screening in sub-Saharan Africa: A randomised trial of VIA vs cytology for triage of HPV-positive women., *Int. J. Cancer.* (2014). doi:10.1002/ijc.29353.
- [68] R. Catarino, P. Vassilakos, S. Scaringella, M. Undurraga-Malinverno, U. Meyer-Hamme, D. Ricard-Gauthier, J.C. Matute, P. Petignat, Smartphone use for cervical cancer screening in low-resource countries: A pilot study conducted in Madagascar, *PLoS One.* 10 (2015) 1–10. doi:10.1371/journal.pone.0134309.
- [69] J. Belinson, Y.L. Qiao, R. Pretorius, W.H. Zhang, P. Elson, L. Li, Q.J. Pan, C. Fischer, a Lorincz, D. Zahniser, Shanxi Province Cervical Cancer Screening Study: a cross-sectional comparative trial of multiple techniques to detect cervical neoplasia., *Gynecol. Oncol.* 83 (2001) 439–444. doi:10.1006/gyno.2001.6370.
- [70] X. Yi, J. Zou, J. Xu, T. Liu, T. Liu, S. Hua, F. Xi, X. Nie, L. Ye, Y. Luo, L. Xu, H. Du, R. Wu, L. Yang, R. Liu, B. Yang, J. Wang, J.L. Belinson, Development and validation of a new HPV genotyping assay based on next-generation sequencing, *Am. J. Clin. Pathol.* 141 (2014) 796–804. doi:10.1309/AJCP9P2KJSXEKCB.
- [71] J.L. Belinson, H. Du, B. Yang, R. Wu, S.E. Belinson, X. Qu, R.G. Pretorius, X. Yi, P.E. Castle, Improved sensitivity of vaginal self-collection and high-risk human papillomavirus

- testing., *Int. J. Cancer*. 130 (2012) 1855–60. doi:10.1002/ijc.26202.
- [72] C.-C. Chang, R.-L. Huang, Y.-P. Liao, P.-H. Su, Y.-W. Hsu, H.-C. Wang, C.-Y. Tien, M.-H. Yu, Y.-W. Lin, H.-C. Lai, Concordance analysis of methylation biomarkers detection in self-collected and physician-collected samples in cervical neoplasm., *BMC Cancer*. 15 (2015) 418. doi:10.1186/s12885-015-1411-x.
- [73] V.M.J. Verhoef, D.A.M. Heideman, F.J. Van Kemenade, L. Rozendaal, R.P. Bosgraaf, A.T. Hesselink, R.L.M. Bekkers, L.F.A.G. Massuger, R.D.M. Steenbergen, P.J.F. Snijders, J. Berkhof, C.J.L.M. Meijer, Methylation marker analysis and HPV16/18 genotyping in high-risk HPV positive self-sampled specimens to identify women with high grade CIN or cervical cancer, *Gynecol. Oncol.* 135 (2014) 58–63. doi:10.1016/j.ygyno.2014.08.003.
- [74] M. Arbyn, P.E. Castle, Offering Self-Sampling Kits for HPV Testing to Reach Women Who Do Not Attend in the Regular Cervical Cancer Screening Program., *Cancer Epidemiol. Biomarkers Prev.* 24 (2015) 769–72. doi:10.1158/1055-9965.EPI-14-1417.
- [75] J.-F. Shi, J.-F. Chen, K. Canfell, X.-X. Feng, J.-F. Ma, Y.-Z. Zhang, F.-H. Zhao, R. Li, L. Ma, Z.-F. Li, J.-B. Lew, Y. Ning, Y.-L. Qiao, Estimation of the costs of cervical cancer screening, diagnosis and treatment in rural Shanxi Province, China: A micro-costing study, *BMC Health Serv. Res.* 12 (2012). doi:10.1186/1472-6963-12-123.
- [76] Y.N. Flores, D.M. Bishai, A. Lorincz, K. V. Shah, E. Lazcano-Ponce, M. Hernandez, V. Granados-Garcia, R. Perez, J. Salmeron, HPV testing for cervical cancer screening appears more cost-effective than Papanicolaou cytology in Mexico, *Cancer Causes Control.* 22 (2011) 261–272. doi:10.1007/s10552-010-9694-3.

- [77] R.P. Insinga, E.J. Dasbach, E.H. Elbasha, A. Puig, L.M. Reynales-Shigematsu, Cost-effectiveness of quadrivalent human papillomavirus (HPV) vaccination in Mexico: A transmission dynamic model-based evaluation, *Vaccine*. 26 (2007) 128–139. doi:10.1016/j.vaccine.2007.10.056.