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Fluorescence microscopy of H&E stained cervical biopsies to assist the diagnosis and grading of CIN

Mario R. Castellanos[a,*], Vijeyaluxmy Motilal Nehru[a], Edyta C. Pirog[b], Lynne Optiz[c]

[a] Division of Research, Department of Medicine, Staten Island University Hospital – Northwell Health, 475 Seaview Ave, Staten Island, NY 10305, USA
[b] Department of Pathology, Weill Cornell Medical College, 525 East 68th Street, New York, NY 10065, USA
[c] Department of Pathology and Laboratory Medicine, Staten Island University Hospital – Northwell Health, 475 Seaview Ave, Staten Island, NY 10305, USA

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ABSTRACT

Background: Prevention of cervical cancer is based upon the accurate diagnosis and grading of cervical lesions identified during screening. The pathological classification of cervical intraepithelial neoplasia (CIN) is problematic, as it relies on subjective criteria and is known to have high interobserver variability and low reproducibility. These limitations can result in either over or under treatment of patients. Biomarkers to improve CIN diagnosis have not overcome all these challenges.

Main body: Here we review the use of a promising optical imaging method using eosin-based fluorescence spectroscopy. This technique is able to perform fluorescent analysis of cervical biopsies directly from hematoxylin and eosin (H&E) stained tissues. Eosin is a brominated derivative of fluorescein. Fluorescence characteristics of protein–eosin complexes can demonstrate tissue changes associated with dysplasia and cancer. In this article we review the progress made towards developing eosin-based fluorescence spectroscopy. We describe the various morphologies seen among the CIN grades with this optical method and highlight the progress made to quantitate the spectral image characteristics.

Conclusion: Eosin-based fluorescence spectroscopy can be used to directly examine H&E stained tissue slides. Relevant areas can be imaged and spectral analysis done to obtain objective data to identify and grade cervical lesions.

1. Background

High-risk types of human papillomavirus (HPV) cause 5% of the world's cancers, which includes cervical, oropharyngeal, anal, penile, and vaginal carcinoma [1]. In less developed regions of the world, cervical cancer remains one of the leading causes of cancer-related deaths in women. In contrast, in developed countries, screening programs are effective in identifying millions of HPV infected women, thereby preventing progression to cervical cancer [2]. Currently, there is no antiviral treatment for chronic cervical HPV infection and cancer is prevented by monitoring those infected with HPV for progression to malignancy. Persistent HPV infection can lead to dysplasia of the cervical epithelium, also known as cervical intraepithelial neoplasia (CIN) [3]. To prevent cervical cancer, surveillance programs center on identifying women that develop high-grade CIN. These lesions are then treated with surgical or ablative therapies. The gold standard for diagnosing CIN and triaging patients for either follow up or treatment is based on histopathology; yet, criteria for identifying and grading CIN are very subjective. This limitation leads to high interobserver variability, with overall low reproducibility and a "flawed" gold standard that potentially can impact clinical care [4,5]. Advances in optic imaging has shown that fluorescence spectroscopy has the ability to detect biochemical and morphological changes that occur as normal cervical tissue progresses through dysplasia to cancer [6,7]. This technology has the capacity to aid standard light microscopy examination of cervical biopsies. In addition, this approach could also have application to other areas of surgical pathology and assist the histopathologic interpretation of other tissue like the breast, thyroid, and skin. In this article we discuss the current understanding HPV carcinogenesis and how it relates to CIN grading. We then review the progress made towards developing eosin-based fluorescence spectroscopy. This imaging technique is able to directly examine hematoxylin and eosin (H&E) stained tissue. We describe the various morphologies seen among the CIN grades with this new imaging method and highlight the potential applications to assist in CIN classification.

Abbreviations: HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; CIN1, CIN1 grade 1; CIN2, CIN2 grade 2; CIN3, CIN1 grade 3

* Corresponding author.
E-mail addresses: mcastellan@northwell.edu (M.R. Castellanos), vmotil2@uic.edu (V.M. Nehru), ecpirog@med.cornell.edu (E.C. Pirog), lopitz@northwell.edu (L. Optiz).

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2. Main text

2.1. Productive and transforming HPV infections

HPV gains entry into mucosal epithelium *via* breaks or traumatized defects that expose the basement membrane. HPV preferentially infects basal keratinocytes that migrate to repair wounded epithelium [8]. Specific receptors for HPV on basal cells are thought to be the cause of this tropism. Once within the cell, the virus can enter into either a non-productive (latent) state or become a productive viral infection. In the latent state, the HPV genome is contained in the nucleus in a free circular form known as an episome [3]. Episomal DNA replication is coupled to the replicative cycle of epithelial cells. When basal epithelial cells enter the differentiation pathway and keratinocytes begin to mature, HPV genes are then transcribed and replicated along with the host cell's DNA. In a latent state, HPV episomal DNA has a very low viral copy in the range of 10–200 copies per basal cell and may exist in this state for years to later become activated into a productive viral infection [9]. In an active viral infection, most HPV DNA replication occurs in the middle and superficial layers of the epithelium via a well-coordinated process of HPV gene expression. The final stages of the viral cycle occur in the superficial layers with L1 and L2 capsid proteins being synthesized to have HPV DNA packaged within the viral capsid to form a complete virion. It is believed that HPV induces cancer in areas where a productive viral infection becomes dysregulated [9]. In high-risk viral HPV types, transformation begins when over-expression of E6 and E7 occurs, interfering with the tumor suppressor proteins p53 and pRb, respectively. Biomarkers used in histopathology attempt to identify these alterations [10,11]. In most cases cervical HPV infections spontaneously become undetectable by all laboratory methods. Clinicians often refer to this as *"clearance"*. Approximately 70% of new HPV infections become undetectable within 1 year and 90% of HPV infections regress without treatment within 2 years. Therefore, about 10% of patients that initially acquire HPV infection will progress to a high-grade CIN [12,13].

2.2. Cervical intraepithelial neoplasia

CIN is characterized by disordered cellular maturation, nuclear atypia, and increased mitotic activity, koilocytic changes, as well as expansion of the zone of proliferating parabasal cells [3]. In CIN grade 1 (CIN1) lesions; the parabasal cells are limited to the lower 1/3rd of the epithelium. In CIN grade 2 (CIN2), the proliferating parabasal cells are located between the lower 1/3 and 2/3^{rds} of the epithelial thickness. While in CIN grade 3 (CIN3) the abnormal expansion of the parabasal cells involves the upper 1/3 of the epithelium [3]. When CIN classification was initially developed, histopathologic grading of the lesion was used to predict biologic behavior. Recent understanding of the intracellular events leading to transformation and progression has led to a better understanding. HPV infections that are productive, leading to complete virion formation, are histologically characterized by minor cellular changes, diagnosed as CIN1 on light microcopy. While, high-grade CIN is caused by a transforming infection, leading to the cellular changes seen in CIN2 and CIN3 morphologies [3,8,9]. These transforming infection results from dysregulated overexpression of HPV E6 and E7, either by a break in HPV E2 during viral integration into the host genome or a disruption of the E2 gene in the episomal viral state [9]. Not all high-grade lesions (CIN 2&3) lesions progress to cancer, as 28% are known to spontaneously regress [14], therefore, biomarkers to assist with CIN diagnosis and predict biologic behavior have been proposed [10,11]. Some of these biomarkers include viral genotype, viral DNA methylation status, immune response markers, and expression of cell cycle regulators like p16, p53, and Rb. Furthermore, CIN2 has been proposed as a heterogeneous category consisting of a productive HPV infection and a true precancer [15]. In the absence of being able to definitely identify which high-grade lesions will progress, the general approach for the clinical management of patients with CIN 2&3 is to treat these women, especially if patient is 30 years or older. Though treatment of CIN has a high success rate, these surgical or ablative therapies significantly increase the risk of future pregnancy complications such preterm delivery and cervical incompetence [16,17].

In addition to not being able to always predict the biologic behavior of CIN lesions, the histopathological classification of cervical biopsies is problematic. This occurs because the criteria for CIN diagnosis is subjective and has high inter and intra-observer variability [18,19]. A study by Stoler et al. showed that the concordance rate of diagnosing CIN1 was 38.2% between community pathologists and an expert panel of gynecological pathologists, while rate for CIN2 was 38% and CIN3 68% [20]. Inaccurate grading of CIN lesions can lead to excessive treatment of patients with low grade lesions or under treatment of high grade lesions potentially risking the development of cervical cancer. The limitations of current cervical cancer screening programs, to accurately diagnose CIN and predict which patients will progress, contribute to the high cost associated with screening and treatment of HPV related cervical disease. In the US this is estimated to be at $4.6 billion [21]. Therefore, it is imperative to continue to increase the ability to identity and grade CIN.

2.3. Ex vivo fluorescence spectroscopy

Optical technologies have the potential to address current limitations of cervical cancer screening programs. Fluorescence spectroscopy can detect biochemical and morphological changes that occur as normal cervical tissue progresses through the various grades of dysplasia to cancer [22,23]. It uses a fluorescent light source to excite tissue that in turn emits fluorescent light at various wavelengths. All cells contain molecules that are capable of emitting fluorescence. Examples of these endogenous fluorophores include NADP, aromatic amino acids, cytokeratins, and various proteins such as collagen and elastin [25,26]. The intrinsic fluorescence of cells and tissues can be seen directly with a fluorescent microscope system and images can then be sent to a computer for a more detailed analysis. As normal cells become dysplastic then cancerous, they undergo metabolic and structural changes that are reflected in the quantity and nature of the endogenous fluorophores present. In addition, the supporting stroma and extracellular tissue also undergoes biochemical and morphological alterations during cancer formation. Changes in collagen type and organization are seen during tumorigenesis [24]. Autofluorescence perturbations of the cervical epithelium and stroma can give rise to specific patterns associated with CIN grades [27]. These observations can be objectively quantified by using image analysis and specific fluorescence signatures can be generated to differentiate normal, precancerous and cancerous tissue [28,29]. These promising studies examining autofluorescence need fresh tissue to be used for optical imaging. This can be a limitation as light microscopy is still the standard and requires biopsy specimens to be stained with hematoxylin and eosin (H&E) for examination. Autofluorescence imaging is done on parallel section and compared with H&E slides.

Another approach that has been used to examine cervical biopsies is fluorescence imaging obtained directly from H&E stained cervical tissue. Eosin is a fluorescent dye, which is a brominated derivative of the well-known compound fluorescein. When eosin complexes with tissue structures, such as proteins, these units produce proportionate changes in eosin-based fluorescence [30–33]. Fluorescence characteristics of H&E stained tissue were described decades ago, to evaluate the skin, pancreas, spleen, colon, and kidney, yet this approach has only recently been applied to cervical biopsies. The advantage of this approach is the ability to use standard H&E-stained tissue sections and analyze the relevant areas of interest seen during conventional light microscopy examination. With fluorescence confocal microscopy, H&E-stained slide can be imaged without any additional processing of the