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# Nicotinamide Phosphoribosyl Transferase a Reliable Marker of Progression in Cervical Dysplasia

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## Abstract

**Background/Aim:** Nicotinamide phosphoribosyl transferase (Nampt) catalyses the rate-limiting step of the mammalian nicotinamide adenine dinucleotide (NAD) salvage pathway. Nampt is highly expressed in several epithelial and mesenchymal neoplasms, where is promotes cell-cycle progression ans chemotherapy resistance. To our knowledge, alterations in Nampt expression have not been examined in cervical intraepithelial neoplasia (CIN) or squamous cell carcinoma (SCC).

**Materials and Methods:** We performed immunohistochemical analysis for Nampt using tissue microarrays on 14 samples of benign cervical squamous epithelium and 15 CIN I, 15 CIN II, and 13 samples of CIN III. The SCCs included 5 low-grade, 67 intermediate-grade, and 81 high-grade tumors.

**Results:** Nampt levels increased with increased CIN grades were compared to benign cervical squamous epithelium. Similarly, Nampt levels increased with increasing SCC grade.

**Conclusion:** Nampt expression is a reliable marker of progression in cervical dysplasia and SCC.

### Keywords

Nicotinamide phosphoribosyl transferase; cervical dysplasia; squamous cell carcinoma

Cervical cancer is the third most prevalent gynecologic malignancy worldwide and more than 85% of the disease burden occurs in developing nations (1–3). Invasive squamous cell carcinomas arising from the surface epithelium are the most common (4). The earliest histological precursor of SCC is low-grade squamous dysplasia (CIN I) characterized by nuclear enlargement, hyperchromasia, and irregular nuclear contours. Higher grades of

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Conflicts of Interest

The Authors report no conflicts of interest.

dysplasia (CIN II-III) are accompanied by increased nuclear-cytoplasmic ratios, coarsening of chromatin, increased mitotic activity, and extension of dysplastic keratinocytes to the upper-third of the cervical epithelium (7). The majority of low-grade lesions regress spontaneously, whereas higher-grade dysplasia further progresses to *in situ* carcinoma, which yet may be curable by surgical management. However, invasive or recurrent disease carries a poor prognosis such that the early detection of cervical cancer and its preceding lesions is vital for optimal patient outcomes (5, 6).

Cervical SCC development is closely associated with persistent high-risk human papilloma virus (HPV) infection (8, 9). The oncogenic potential of HPV comes largely from two viral proteins, *E6* and *E7*, that bind many cellular targets, including the p53 and pRb proteins resulting in their degradation (10–13). *E6* and *E7* function coordinately to ablate apoptotic and cell-cycle checkpoint responses, increases telomerase activity, and increases somatic mutations, all of which promote carcinogenesis (6, 10–15). HPV infection also promotes carcinogenesis by increasing STAT3 protein kinase expression and activity (16). Not surprisingly, CIN shows increased STAT3 protein with increasing CIN grade and in cervical SCC compared to benign cervical epithelium (17). STAT3 activity induces higher expression of nicotinamide phosphoribosyl transferase (Nampt), an enzyme that catalyses the rate-limiting step of nicotinamide adenine dinucleotide ($NAD^+$) synthesis (18). Nampt is elevated in a number of human malignancies, including gliomas, melanoma, prostate, breast, ovarian, thyroid, and colorectal carcinomas (19–26). High Nampt expression promotes cell growth and survival, DNA synthesis, mitochondrial biogenesis, angiogenesis, contributing to malignant progression (27). Recently Nampt protein expression was found to be increasingly elevated with higher grades (dedifferentiation) of leiomyosarcomas and aggressive histologic subtypes of rhabdomyosarcomas (28).

Based upon these observations we hypothesized that Nampt expression would be increasingly elevated with higher grades of *in situ* cervical dysplasia and squamous cell carcinoma as compared to benign cervical epithelium. Herein we performed a semi-quantitative immunohistochemical analysis of Nampt expression utilizing a tissue microarray.

## Materials and Methods

Tissue microarrays (TMAs), catalog numbers CIN481 and CxC1501, were purchased from US Biomax, Inc. (Rockville, MD, USA). Together the TMAs contained the following: 14 samples of benign squamous epithelium, 15 CIN I, 15 CIN II, and 13 samples of CIN III. Tissue samples of squamous cell carcinoma included 5 low grade, 67 intermediate grade, and 81 high-grade tumors. Additionally 13 cores of benign endocervical columnar epithelium and 7 samples of endocervical adenocarcinoma were represented in the microarrays. The biopsy diameters of each core were 1.5 mm and 1.1 mm in the CIN481 and CxC1501 TMAs respectively.

### NAMPT immunohistochemistry (IHC).

The concentration of primary Nampt antibody was optimized to normal kidney as control tissue. The staining of the TMA was performed in the Tissue Core Histology Lab Facility at

the Moffitt Cancer Center. The microarray slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ, USA) as per the manufacturer's protocol with proprietary reagents. Briefly, slides were deparaffinized on the automated system with EZ Prep solution (#950-100; Ventana Medical Systems). The heat-induced antigen retrieval method was used in Cell Conditioning 1 (#950-124; Ventana Medical Systems). Mouse monoclonal antibody to human Nampt (#ALX-804-717; Enzo life Sciences, Plymouth Meeting, PA, USA) was used at a 1:1,000 concentration in Dako antibody diluent (#S0809; Dako, Carpenteria, CA, USA) and incubated for 60 min. The Ventana anti-mouse or rabbit secondary antibodies were used for 16 min. The detection system used was the Ventana OmniMap kit. Slides were then dehydrated and cover-slipped as per standard laboratory protocol.

### Evaluation of NAMPT staining.

Relative Nampt protein expression was determined as immunostain intensity scored on a 0-3 scale as follows: no staining as 0, light staining as 1, moderate staining as 2, and heavy staining as 3. The percentage of cells stained was measured, with no detectable staining as 0, 1-33% as 1, 34-66% as 2 and 67-100% as 3. The final IHC score was the product of the percentage of cells stained multiplied by the intensity score, allowing for a maximal score of 9 and a minimal score of 0. Nuclear and cytoplasmic Nampt staining was seen in all tissue samples examined, although at low levels in benign squamous and columnar epithelium. We therefore measured and quantified Nampt staining in the nuclear and cytoplasmic compartments. Since even experienced pathologists can disagree on diagnosing the lower dysplasia grade, all cores on the TMA slide were reviewed by three pathologists and to ensure that the histologic CIN grading was exact and accurate.

### Statistical analysis.

The standard error of the mean (SEM) IHC score was calculated by using the standard deviation for the staining scores of each tumor type and dividing this number by the square root of the sample size.

## Results

Following IHC processing, due to tissue loss we were left with 14 samples of benign squamous epithelium and 13 cases of benign endocervical columnar epithelium. Of the CIN tissue 11 CIN I, 9 CIN II, and 8 III samples cases were available for analysis. Of the invasive carcinoma cases 5 low grade, 56 intermediate grade, and 53 high-grade SCC, and 7 cervical adenocarcinomas tissue cores were available for analysis. A number tissue samples on the TMA were not analyzed as they were unrelated to purpose of the present study (adenosquamous carcinoma-7 cases, CxC1501) or insufficiently represented (hyperplasia-2 cases CxC1501, mucinous adenocarcinoma-1 case CIN481). Examples of Nampt IHC of benign, dysplastic, and malignant tissues are shown in Figure 1. The number of cases examined and the quantified IHC scores for each tissue type are given in Table I.

## Discussion

In cervical biopsies CIN is commonly analyzed by the microscopic examination of properly oriented hematoxylin and eosin stained tissue sections (29–33). Commonly, these analyses are supplemented by p16 immunohistochemistry that is a sensitive marker for HPV E7 protein expression (29, 30). Cushieri *et al.* (30) reviewed nine studies including 2,178 cases and found p16 expression in 7% of benign cervical epithelium, and in 54%, 86%, and 96% of CIN I-III cases, respectively. Other less often used protein markers for CIN include Ki-67, telomerase, p63, and E-cadherin (29–33). Here we show that Nampt is increased in a grade-dependent manner in CIN and cervical SCC. Nampt catalyses the rate-limiting step of $NAD^+$ synthesis and Nampt levels and enzymatic activity are the main determinants of cellular $NAD^+$ levels (27, 33, 34). $NAD^+$ is both a vital energy source and signal transduction molecule that is rapidly degraded in malignant cells, hence Nampt protein levels and Nampt-mediated $NAD^+$ synthesis is increased in many malignancies (19–27, 35). Nampt is most highly expressed in active dividing cells and higher Nampt expression confers a worse prognosis in prostate and gastric cancers and increases chemotherapy resistance in gastric cancer (22, 25, 27). Additionally, Nampt is more highly expressed with increasing Fuhrman grade in renal cell carcinoma, and in higher histiologic grades of oral squamous cell carcinoma and leiomyosarcomas (28, 36, 37).

In the present case of invasive cervical SCC we were also able to demonstrate that Nampt i) is a marker for cervical dysplasia in CIN which increases in a grade-dependent manner, ii) increases with higher grades of cervical SCC, and iii) is highly expressed in cervical adenocarcinoma compared to benign cervical columnar epithelium (Table I and Figure 1) (19–32, 38). Also since p16 is expressed in only 54% of CIN I, and we found Nampt expression to be doubled in CIN I as opposed to benign squamous epithelium (Table I), our study suggests that Nampt IHC may be useful in CIN analysis in these situations. This is noteworthy particularly since $NAD^+$ synthesis is obligatory for cell survival and growth (27, 29, 30, 39). Further investigation is needed to ascertain the possible use of Nampt IHC in the analysis and diagnosis of CIN.

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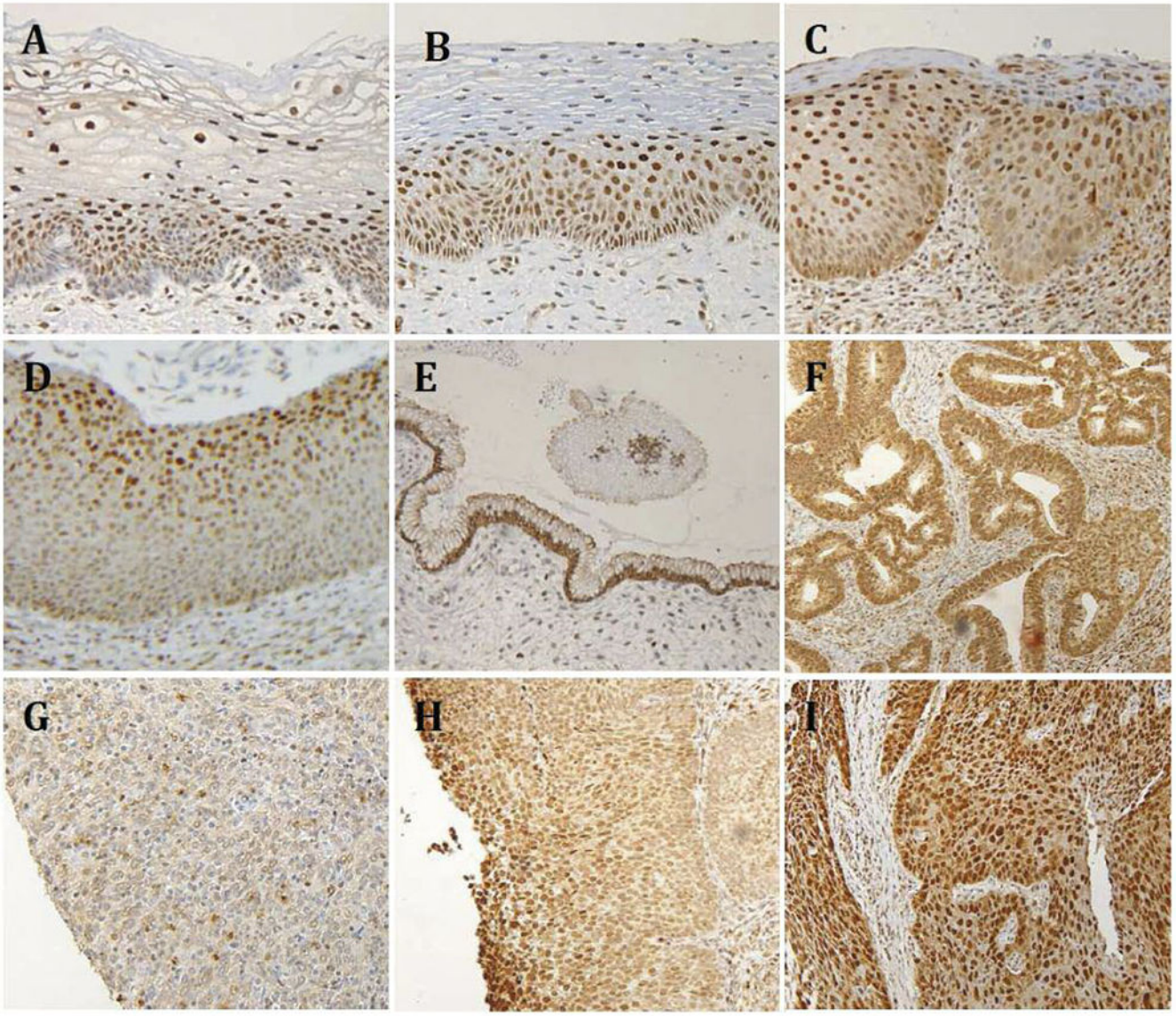


**Figure 1.**

High- and low-power views of Nampt IRC of benign cervix (A), CIN I (B), CIN II (C), CIN III (D), benign columnar epithelium (E), adenocarcinoma (F), grade ISCC (G), grade II SCC (H), and grade III SCC (I). Panels A-D are high-power views (200x) and E-I are low power views (100×).

**Table I.**

IHC: Relative Nampt staining in the tissue microarrays comparing benign cervical tissue to CIN I-III and grades I, II, and III SCC.

| Tissue Type | Sample number | Average nampt IHC score | SEM |
|---|---|---|---|
| Benign cervical squamous epithelium | 14 | 1.57 | 0.14 |
| CIN I | 11 | 3.45 | 0.49 |
| CIN II | 9 | 3.86 | 1.02 |
| CIN III | 8 | 4.91 | 0.76 |
| SCC Grade I | 5 | 4.00 | 0.55 |
| SCC Grade II | 56 | 4.91 | 0.32 |
| SCC Grade III | 53 | 6.43 | 0.37 |
| Benign cervical columnar epithelium | 13 | 1.77 | 0.23 |
| Adenocarcinoma | 7 | 7.71 | 0.95 |

IHC: Immunohistochemistry; SEM: standard error of the mean.