UPDATE ON MICRONUTRIENTS AND CERVICAL DYSPLASIA

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This manuscript reviews the current state of knowledge of cervical carcinogenesis and present recent results and introduces ongoing studies on the relationships among micronutrients and natural history of high-risk human papillomaviruses (HR-HPVs) and cervical intraepithelial neoplasia (CIN). Numerous studies have attempted to determine associations between micronutrients and risk of CIN and cervical cancer. Studies that were conducted before a reliable test for assessing HPV infections was available may have resulted in misclassification because of differences in assay sensitivity, which could have led to residual confounding. Another limitation in previous studies may be related to methodologic limitations such as the proper choice of controls for case-control studies. Since cervical cancer does not develop in the absence of HR-HPV infections, only controls exposed to HR-HPV should be included in studies that investigate cofactors for CIN or cervical cancer. Also, the recruitment of subjects for these studies had been based on screening programs that used different approaches such as cytology, colposcopic impression, or biopsy to identify pre-neoplastic cervical lesions. Recent studies have demonstrated that some of these approaches could lead to substantial underdetection and misclassification of preneoplastic lesions of the cervix. Recent studies that addressed these issues have demonstrated that folate is an important micronutrient in cervical cancer prevention via its influence on HR-HPV and the development of CIN. Carefully designed ongoing studies are expected to generate data on whether folate-related biomarkers could be used to identify subjects who are at risk of developing cervical cancer and whether folate supplementation will be beneficial in preventing cervical cancer in women exposed to HR-HPV. (*Ethn Dis*. 2007;17[suppl 2]:S2-14–S2-17)

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Many micronutrients may play an important role in cancer prevention because of their critical roles in free radical scavenging, DNA synthesis/repair, and maintenance of DNA methylation patterns. The cancer-protective role of vitamins A and E is likely to be through their effects on cell differentiation and proliferation. Specific micronutrients may modify cervical carcinogenesis by their influence on the natural history of cancer-associated or high-risk (HR) human papillomaviruses (HPVs), the main risk factor for cervical intraepithelial neoplasia (CIN) and invasive cervical cancer. This manuscript reviews the current state of knowledge of cervical carcinogenesis, presents recent results on the relationships between micronutrients and cervical cancer and HPV, and introduces some of the ongoing studies in this area.

Biologically, cervical cancer arises as a result of an accumulation of damages to cell cycle control and genomic instability. Since genomic instability and risk of cancer in general are likely to be modified by micronutrient status, numerous studies have attempted to determine associations between micronutrients and risk of cervical cancer or CIN. Early epidemiologic studies that focused on diet and cervical cancer focused mainly on invasive disease; these studies have been reviewed by Potischman and Brinton.[1] According to this review, low vitamin C and carotenoid status were associated fairly consistently with both cervical cancer and CIN, whereas results for vitamin E status are less consistent, and the effect of folate status may be restricted to early CIN. These studies were limited by inadequate HPV measures, which may have compromised the interpretations of findings. Recent nutritional studies on cervical cancer and CIN have taken HR-HPV infections into account and have attempted to determine if previously suspected nutritional factors act as independent risk factors or as cofactors of HPV and HPV-related factors. A comprehensive review of studies conducted after taking HPV into account is published by Garcia-Closas et al.[2] These authors concluded that the available evidence for an association between diet and nutritional status and cervical carcinogenesis, taking HPV infection into account, is not yet convincing for occurrence or regression of CIN. However, these studies have attempted to address the nutritional influences on some aspects of the natural history of HPV, mainly HPV persistence. Since nutritional status is likely to play a role in early cervical disease, the attempt to evaluate the association between nutrients and HPV was a step forward in this research area. Unfortunately, these studies were short-term and assessed only a limited number of nutrients and therefore have produced inconsistent results: higher concentrations of trans- and cis-lycopene reduced the time to clearance of oncogenic HPV infections in US women;[3] lower serum β-carotene, β-cryptoxanthin, and lutein concentrations were associated with HPV persistence among US Hispanics;[4] and low plasma vitamin B-12 concentrations were associated with persistent HPV infection among Hispanic women.[5] A small study among Hispanic women, however, did not support a role for folate, vitamin B12, or homocysteine in HPV persistence or cervical dysplasia.[6] Palan et al found no associations among circulating concentrations of retinal, α- and β-carotene, or lycopene and persistent HPV infections.[7] The aforementioned studies were not only short term (3–10 months) but also only tested their study subjects for HR-HPV at two time points and focused on one aspect of the natural history of HR-

HPV (persistence or clearance) in a given study.

We recently completed a study intended to be the first comprehensive, long-term (24-month), prospective, follow-up evaluation of the influence of folate on three aspects of the natural history of HR-HPV. The study was designed to evaluate the associations between folate and 1) the incidence of HR-HPV infections; 2) persistence of HR-HPV; and 3) clearance of HR-HPV after controlling for other specific micronutrients (vitamins B-12, C, A, E, and carotenoids). We demonstrated that higher blood concentrations of folate are associated with a lower likelihood of becoming HR-HPV-positive and of having a persistent HR-HPV infection and, in infected women, a greater likelihood of clearing HR-HPV. These associations held after controlling for other micronutrients and other known risk factors for cervical cancer,[8] which strongly suggests a role for folate in early cervical carcinogenesis.

Folate could modify the risk associated with HR-HPV in several ways. Reduced immunocompetence associated with folate deficiency[9,10] could increase the risk of infection with multiple types or higher viral loads of HR-HPV. This increased risk is likely to increase the acquisition of high-risk HR-HPV types, their persistence, and their integration into the host genome. A common chromosomal fragile site that is sensitive to folate deficiency coincides with a site of HPV-16 integration in the tissues of primary cervical carcinomas[11] and three of the four sites at which HPV-18 integrates its DNA into the host,[12] which suggests a plausible mechanism through which suboptimal folate concentrations could increase the risk for cervical cancer. Although the mechanisms of HPV integration and cervical carcinogenesis are poorly understood, HPV DNA that remains episomal may be more likely to be expelled, which may result in clearance of HPV infection. A decrease in persistent HPV infection and increased clearance of HPV in subjects with high folate may result from preventing its integration. Recent studies also suggested that folate deficiency induced upregulation of folate receptor/heterogeneous ribonucleoprotein E1 (hnRNP-E1) may modulate HPV viral proliferation in the cervix.[13]

On the basis of folate's effects on the natural history of HR-HPV, we have also hypothesized that supplementation with folic acid may enhance HR-HPV clearance. We are investigating the effect of supplementation with folic acid on clearance of HPV 16 and other HR-HPV by conducting a 12-month, double-blind, placebo-controlled trial of supplementation with 5 mg of folic acid per day (R01 CA102489-PI, Piyathilake). This study is also designed to generate data on the mechanisms of action of folic acid on the behavior of HR-HPV, which will aid greatly in understanding the mechanisms of actions of folate in cervical carcinogenesis.

As discussed earlier, no convincing reports suggest associations between nutritional factors and the occurrence of CIN. Although these studies have taken HPV infection status into account, concerns have been raised about what techniques are used to generate HPV results and how these HPV data are handled in the statistical analysis. No standardized method for detecting HPV exists, but several HPV test systems are in use in the United States. So far, the Food and Drug Administration has approved one, the Hybrid Capture assay (HC-2), which targets 13 HR-HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The literature demonstrates that 15 HPV genotypes, HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82, are considered carcinogenic or HR-HPV types. Three other HPV types, HPV 26, 53, and 66, are considered probably carcinogenic or probably high risk.[14] The HC-2 assay test uses a combination of DNA probes to detect 13 HR-HPV genotypes and therefore does not give type-specific HPV results. L1 consensus PCR-based assays most commonly detect the several HPV types, including HPV 16, 14 other known types of HR-HPV (18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), and three possibly high-risk HPV genotypes (HPV 26, 53, and 66). Most nutrition studies have tested for HPV using the HC-2 assay and therefore may have missed HPV type-specific nutrient effects.

Infection with HR-HPV is a necessary cause in the development of CIN 2 or 3, which makes infection with HPV an intermediary causal factor of CIN 2 or 3. Given this information, in examining the association of nutrients and CIN, analysis should be restricted to HR-HPV-positive women because controlling for HPV infection as a confounder could erroneously nullify the associations of interest.[15] Most studies have included both HR-HPV-positive and HR-HPV-negative women in nutrition studies and have controlled for HPV as a confounding variable.

The other major limitation in most studies is related to inaccurate case definition protocols (eg, based only on cytology or colposcopic impressions rather than biopsy) that can lead to substantial underdetection and misclassification of preneoplastic lesions of the cervix.[16,17] Among available diagnostic methods, histologic diagnoses based on cervical biopsy samples provide the most accurate clinical diagnosis of a cervical lesion. According to histologic classification, CIN lesions are categorized into 3 groups, $\leq$ CIN 1, CIN 2, and CIN/CIS. Among these, $\leq$ CIN 1 lesions are not considered to be truly preneoplastic, whereas the $\geq$ CIN 2 lesions are considered to be true preneoplastic lesions of the cervix. According to cytologic diagnosis, cervical squamous intraepithelial lesions (SILs) are subdivided into atypical squamous cells of undetermined significance (AS-

Table 1. Histological diagnoses of abnormal Pap tests. Results from the University of Alabama study population.

	Histologic Diagnosis			
Pap Diagnosis	**Normal or Reactive**	**CIN 1**	**CIN 2**	**CIN 3**
ASCUS	14%	62%	14%	10%
LSIL	34%	43%	15%	8%
HSIL	19%	23%	36%	22%

CIN=cervical intraepithelial neoplasia; ASCUS=atypical squamous cells of undetermined significance; LSIL=low-grade squamous intraepithelial lesions; HSIL=high-grade squamous intraepithelial lesions.

CUS), low-grade and high-grade SILs (LSIL and HSIL, respectively). Most studies have made the assumption that LSIL is the cytologic equivalent to CIN 1, HSIL is the cytologic equivalent to histologic CIN 2 or 3, and CIS and both LSIL and HSIL are treated as cases that have preneoplastic lesions. However, as shown in Table 1, if biopsy diagnosis is available for women with cytologic diagnosis, it is clear that most women with diagnosis of LSIL and HSIL only have $\leq$ CIN 1 lesions, which are not considered to be true preneoplastic lesions. Also, a portion of ASCUS is likely to have true preneoplastic lesions.

We addressed some of these limitations in our recent studies. We examined whether specific micronutrients play an independent role in the development of CIN $\geq$ 2 (based on biopsy diagnoses) among HR-HPV-positive women enrolled in an observational study (Atypical Squamous Cells of Undetermined Significance-Low-Grade Squamous Intraepithelial Lesion (ASCUS-LSIL) Triage Study – ALTS) conducted by the National Cancer Institute. This study assessed cervical lesions by employing a rigorous biopsy-based review protocol, used sensitive HPV testing techniques (Hybrid Capture and PCR), measured several micronutrients (folate; vitamin B-12, A, C, and E; and total carotene) in blood, and used a comprehensive questionnaire to gather information on risk factors for CIN (age, race, contraceptive use, smoking, number of sexual partners, age at first intercourse and parity). We observed that 1) women with lower erythrocyte folate who were positive for any HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) were two times more likely to have $\geq$ CIN 2; 2) women with lower erythrocyte folate who were positive for HPV 16 were 9 times more likely to have $\geq$ CIN 2; and 3) other risk factors were not independent risk factors for CIN, and other micronutrients were also not independent risk factors for CIN. These observations strongly suggested that folate plays an important role in cervical carcinogenesis, and this protective effect is likely to be HPV type-specific. Two chemoprevention trials[18,19] that assessed the effect of oral supplementation with folic acid in CIN women concluded that folic acid does not enhance the regression of early epithelial lesions. These clinical trials recruited subjects with established cervical dysplasia and concluded that folate has no significant effect on the regression of established cervical lesions. Thus, the results of these studies suggest that folic acid supplementation is not an effective therapeutic approach, but they do not provide any evidence against the hypothesis that increased folate levels in HR-HPV-positive women may prevent the development of preneoplastic lesions of the cervix.

We are currently also investigating (R01CA 105448-PI, Piyathilake) whether folate-related biomarkers such as alterations in DNA and histone methylation and DNA damage markers in cervical cells can be useful in identifying underlying precancerous lesions and whether these markers can be used to identify women who are at risk of developing CIN because of their exposure to HR-HPV. Results from this study are expected to be reported in 2008 or 2009. These biomarker-based studies are important because, to date, no reliable way exists to predict the progression potential of HR-HPV-exposed cervical cells to CIN 3 or invasive cancer. As a consequence, overtreatment is considerable, which represents both an unnecessary burden for women participating in cervical screening programs and additional healthcare costs. Identification of new molecular markers may help determine the prognosis of CIN and to change treatment algorithms. We believe that status of DNA damage and the alterations in methylation of DNA (global, CpG island and gene-specific) and histones are important markers to be validated because of their significance in the process of cervical carcinogenesis and the possibility of reversing their status by nutritional or other therapeutic interventions. The samples collected in this study, other than biopsies, are non-invasive, present minimum subject burden, and are logistically feasible on a large scale. Evaluation of biomarkers in more accessible tissues (eg, cervical brush samples) and in target tissues (eg, biopsies) will allow us to see whether changes in more accessible tissues reflect changes in target tissues. The study intends to validate biomarkers that are feasible and cost-effective to perform on a large scale as well. The prospective follow-up design will allow for measurement of markers before the development of outcome. This will allow determination of risk progression profiles based on differences in these

markers. In addition, we will be measuring the same markers and other risk factors through the study period to determine any changes that take place in the markers and the relationship of those changes to progression from low-grade lesions to high-grade lesions. To our knowledge, this is the first study designed to prospectively explore the potential use of these markers along with folate status as potential intermediate markers for cervical cancer risk.

In summary, recent studies that are conducted with proper control for HR-HPV and other risk factors for cervical cancer have demonstrated that folate is an important micronutrient in cervical cancer prevention via its influence on HR-HPV. Carefully designed ongoing studies are expected to generate data on whether folate-related biomarkers could be used to identify subjects who are at risk of developing cervical cancer and whether folate supplementation will be beneficial in preventing cervical cancer in women exposed to HR-HPV.

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AUTHOR CONTRIBUTIONS

Design concept of study: Piyathilake
Acquisition of data: Piyathilake
Data analysis and interpretation: Piyathilake
Manuscript draft: Piyathilake
Statistical expertise: Piyathilake
Acquisition of funding: Piyathilake
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