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MICRONUTRIENTS AND CERVICAL NEOPLASIA — RECENT ADVANCES IN RISK ASSESSMENT

CHANDRIKA J. PIYATHILAKE*

DEPARTMENT OF NUTRITION SCIENCES OF THE UNIVERSITY OF ALABAMA AT BIRMINGHAM, BIRMINGHAM, ALABAMA, USA

REVIEW

ABSTRACT. THIS MANUSCRIPT reviews the current state of knowledge of cervical carcinogenesis and present recent results on the relationships among micronutrients and natural history of high-risk human papilloma viruses (HR-HPVs), genetic polymorphisms and cervical intraepithelial neoplasia (CIN). Numerous studies have attempted to determine associations between micronutrients and risk of CIN and cervical cancer. Studies conducted before a reliable test for assessing HPV infections was available may have resulted in misclassification due to differences in assay sensitivity leading potentially to residual confounding. Another limitation in previous studies may be related to methodological 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 ideally be included in studies that investigate co-factors 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 clearly demonstrated that some of these approaches could lead to substantial under detection 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. Future studies are warranted to assess whether folate-related biomarkers may be used to identify subjects who are at risk of developing cervical cancer. Further studies are also needed to systematically evaluate the interactions between folate and other micronutrients and polymorphisms in the folate metabolic pathway enzymes.

*ADDRESS ALL CORRESPONDENCE TO: DR. CHANDRIKA J. PIYATHILAKE, DEPARTMENT OF NUTRITION SCIENCES, DIVISION OF NUTRITIONAL BIOCHEMISTRY AND MOLECULAR BIOLOGY, UNIVERSITY OF ALABAMA AT BIRMINGHAM, WEBB 318A, BIRMINGHAM, AL 35294, USA.
TELEPHONE: 205-975-5398. FAX: 205-966-2859. E-MAIL: piyathic@uab.edu

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1. INTRODUCTION

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. 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. Genetic polymorphisms related to micronutrient pathways may also modify cancer risk and it is likely that gene-nutrient interactions between the two may further modify the risk of specific cancers or precancerous lesions. This manuscript reviews the current state of knowledge of cervical carcinogenesis and present recent results on the relationships between micronutrients and natural history of HR-HPVs, genetic polymorphisms and CIN.

Cervical cancer is one of the most common malignancies in women. Worldwide, cervical cancer is second only to breast cancer as the most common malignancy in women, considering both incidence and mortality [1,2]. In the United States, it accounted for an estimated 12,900 new cases and 4,400 deaths in 2001 [3]. In much of the world, cervical cancer is diagnosed in women in their 30s and has a major impact on the stability and social adhesion of families. The prevalence of cervical cancer is predicted to increase over the next several decades as women in the developing world begin to age and as the number of women infected with human immunodeficiency virus (HIV) increases [4]. CIN, which precede cervical cancer, have also reached epidemic proportions, with an estimate of at least 600,000 new cases per year [5].

2. HPV AND CERVICAL CARCINOGENESIS

Epidemiological and molecular studies have shown a causal relation between infection with HR-HPV and cervical cancer [6,7]. Studies that used improved HPV testing procedures have established HR-HPV as a causative agent in CIN as well [8]. Therefore, one approach for preventing CIN and cervical cancer could be by developing a HPV vaccine and they are likely to be commercially available in the next several years [9,10]. The addition of a vaccine against HPV-16, the most common HR-HPV type found in screening in the United States, was projected to be a cost-effective use of health care resources [11]. Studies predict that a type-specific HPV vaccine may reduce, but not eliminate the risk of cervical cancer [12,13]. Because of inadequate data on its long-term effectiveness, impact of type specific vaccines on other HR-HPV and duration of immunity, it is unlikely that routine screening programs or any other preventive measures will be replaced with HPV vaccines in the near future. Also, vaccines will not take care of millions of women already infected with HR-HPV, women who are likely to get infected with HR-HPV types other than the specific HPV types vaccines are focused on, or women who do not have access to vaccines. Therefore, other means of HPV control should be considered in the management of HPV related risk of cervical cancer.

The high prevalence of HPV infection relative to the low incidence of invasive cervical cancer and CIN suggests that the vast majority of HPV infections do not lead to these lesions and other co-factors, including nutritional status may modulate the natural history of HR-HPV and progression of HR-HPV infections to CIN and cervical cancer. There is evidence that most HPV infections are transient, and only women who harbor a persistent HR-HPV infection are likely to develop cervical lesions [14]. Although HPV persistence is an important aspect in the natural history of HPV, understanding the other aspects of the natural history of HPV such as acquisition and clearance is important in reducing the overall HPV associated risk of cervical cancer. Acquisition of an oncogenic HPV type and its reduced clearance increases the likelihood of persistent infection which may lead to viral integration and neoplastic progression.

3. ASSOCIATION BETWEEN MICRONUTRIENTS AND NATURAL HISTORY OF HR-HPV

Several short-term studies assessing limited numbers of nutrients to evaluate the significance of micronutrients in the natural history of HPV have resulted in inconsistent results. Effects of higher concentrations of trans- and cis-lycopene in reducing the time to clearance of oncogenic HPV infections in US women [15], an association between lower serum β-carotene, β-cryptoxanthin, and lutein concentrations and HPV persistence among US Hispanics [16], and an association between low plasma vitamin B_{12} concentrations and persistent HPV infection among Hispanic women [17] have been reported. A small study among Hispanic women, however, did not support a role for folate, vitamin B_{12}, or homocysteine in HPV persistence or cervical dysplasia [18]. Palan et al. found no associations among circulating concentrations of retinal, α- and β-carotene or lycopene and persistent HPV infections [19]. The aforementioned studies were shorter term (3–10 months) and 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. These individual studies were also not designed to investigate a combination of anti-oxidants, folate and vitamin B_{12} in order test the relationship between specific micronutrients and the natural history of HR-HPV after controlling for confounding by other important micronutrients.

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 the natural history of HPV. The study used the HC-2 assay to classify subjects as positive or negative for HR-HPV. The study was designed to evaluate the associations between folate and the (i) likelihood of becoming HC-2 test-positive (incidence of HR-HPV); (ii) repeated HC-2 positive test (persistence of HR-HPV); and 3) likelihood of becoming test-negative after an infection (clearance of HR-HPV) after controlling for other specific micronutrients (vitamins B-12, C, A, E and carotenoids). We demonstrated that higher folate status was significantly and inversely associated with becoming positive for HR-HPV and with positive test status. Higher folate status was also positively associated with becoming test negative. These associations held after controlling for other micronutrients and other known risk factors for cervical cancer [20].

4. BIOLOGICAL PLAUSIBILITY FOR ASSOCIATIONS AMONG FOLATE AND NATURAL HISTORY OF HPV

Folate could modify the risk associated with HR-HPV in several ways. Reduced immunocompetence associated with folate deficiency [21,22] could increase the risk of infection with multiple types or higher viral loads of HR-HPV. This 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 has been shown to coincide with a site of HPV-16 integration in the tissues of primary cervical carcinomas [23] and three of the four sites at which HPV-18 integrates its DNA into the host [24], suggesting a plausible mechanism through which suboptimal folate concentrations could increase the risk for cervical cancer. In contrast to advanced lesions and cervical cancer [25], integration of HPV DNA into the host genome has been considered a rare event in early pre-neoplastic lesions of the cervix. Recent studies that used novel quantitative real-time PCR techniques, however, have demonstrated that integration of HPV may occur in early CIN lesions [26] and even in those containing HPV but no CIN [27]. Also, rapid progression, in 1 to 2 years, from non-CIN lesions or CIN 2 to CIN 3 was shown to be associated with a heavy load of integrated HPV. Although the mechanisms of HPV integration and cervical carcinogenesis are poorly understood, it is possible that HPV DNA that remains episomal would be more likely expelled, resulting 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.

5. ASSOCIATION BETWEEN MICRONUTRIENTS AND CERVICAL CARCINOGENESIS

Over the last several decades, numerous studies have attempted to determine associations between micronutrients and risk of CIN and invasive cervical cancer. Unfortunately, most of these studies were conducted before a reliable test for assessing HPV infections were available. There could have been misclassification due to differences in assay sensitivity leading potentially to residual confounding. Another limitation in previous studies may be related to methodological 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 ideally be included in studies that investigate co-factors 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 clearly demonstrated that some of these approaches could lead to substantial under detection and misclassification of preneoplastic lesions of the cervix [28,29]. Previously conducted studies with these limitations have demonstrated that increased risk for cervical cancer has been consistently associated with behavioral factors traditionally associated with sexually transmitted diseases, including early age at first intercourse and multiple sex partners [30], poor hygiene [31], low socioeconomic status [32], and race [33]. Further, increased risk for cervical cancer also has been consistently associated with behavioral factors that are not traditionally associated with sexually transmitted diseases, including smoking [34], oral contraceptive use, and micronutrient deficiencies including vitamin C [35], β-carotene [36,37], and folate [38]. Whether these risk factors play an independent role or interact with HPV in the development of CIN had remained unclear. Two case control studies have demonstrated that dietary and blood folate levels are inversely associated with risk of CIN, either independently [39] or in interaction with HPV-16 [40]. Two folic acid supplementation trials, however, showed no significant effect of folic acid on rates of CIN regression [41,42]. Two β-carotene chemoprevention trials suggested that this micronutrient also might not prevent cervical cancer [43,44]. None of these studies exclude the possibility that these micronutrients may prevent the progression of HR-HPV positive low-grade cervical lesions to high-grade cervical lesions or cervical cancer. A recent case-control of study of risk factors for invasive cervical cancer among women exposed to cancer-associated HPVs reported that smoking is associated with increased risk and black race was associated with decreased risk of cervical cancer. In the same study, oral contraceptive use was unrelated to the risk of cervical cancer and multi-parity was only weakly related to risk

6. BIOLOGICAL PLAUSIBILITY FOR ASSOCIATIONS AMONG FOLATE AND CIN

Mechanistically, folate is likely to be associated with CIN in several ways in addition to its effects on the natural history of HR-HPV. Folate deficiency leads to DNA and chromosomal instability, risk factors for cancer, by several mechanisms including uracil misincorporation [45,46], impaired DNA excision repair, and suboptimal cellular DNA repair capacity. Supplementation with folic acid minimizes these DNA and chromosomal changes [47]. Intervention studies in humans taking folate supplements have shown that DNA damage in peripheral blood cells is minimized when plasma concentrations of these vitamins are high [46]. Further, DNA instability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes in vitro [45]. Folate deficiency also modulates DNA repair, DNA strand breakage, and uracil misincorporation in immortalized human colonocytes [48].

Markers of DNA or chromosomal instability as discussed below are seen in CIN and cervical cancer. Chromosomal instability develops at early

stages of cervical neoplasia and can be detected even in premalignant lesions and these changes are thought to arise as a direct consequence of infections with HR-HPVs [49]. Genomic instability may contribute importantly to the rapid selection of clonal cell populations that are able to overcome various environmental challenges that arise during cervical carcinogenic progression [50]. In this light, genome instability could be described as "an enabling characteristic" of affected cells. Presence of micronuclei, a measure of both chromosomal breakage and chromosomal loss [51] is shown to be higher in patients with cervical cancer and CIN [52,53]. The latter study also reported that smokers with CIN have higher numbers of micronuclei compared to non-smokers with similar lesions. A stepwise increase in the frequency of micronuclei has also been reported along the spectrum from CIN lesions to cervical cancer, suggesting that detection of micronuclei may be a useful marker of cervical cancer risk [54]. Another plausible mechanism through which folate deficiency might increase risk for cancer is related to its effects on DNA methylation. We have shown that both serum and cervical tissue folate levels are significantly correlated with cervical DNA methylation level [55], suggesting that folate deficiency may result in methyl insufficiency in the cervix. There is also evidence that viral infection induces hypomethylation of DNA as it initiates cell transformation [56]. Kim et al. reported incremental prevalence of global DNA hypomethylation in cervical biopsies along the spectrum from normal to CIN and cancer [57].

Since folate is directly involved in DNA synthesis, DNA repair and DNA methylation, it is biologically highly plausible to see an interaction between folate, HPV and risk of CIN. Individuals with higher folate status are very likely to assist in repairing DNA instability or improving DNA hypomethylation caused by HPV infections which in turn may reduce viral persistence and integration. These likely mechanistic relations among folate, HPV and risk of CIN suggests that folate's effects of HPV clearance or in preventing the progression of HPV associated low-grade cervical lesions to high grade lesions is likely to be more effective when the HPV and associated lesions are not well established or when the lesions are not true preneoplastic lesions (≤ CIN 1).

7. ASSOCIATION BETWEEN MICRONUTRIENT-RELATED POLYMORPHISMS AND CERVICAL CARCINOGENESIS

Tetrahydrofolate (THF) serves as a carrier of active carbon groups that lead primarily to cellular methylation reactions and the synthesis of nucleotides. Aberrations in these two reactions are thought to contribute to the etiology of cancer by effecting genomic stability and the regulation of gene transcription. In the folate metabolic pathway, methylenetetrahydrofolate reductase (MTHFR) irreversibly commits 1-carbon units toward the methylation reactions and away from synthesis of DNA. Methylation of DNA, which occurs at the cytosine residues of CpG dinucleotides by an enzymatic reaction that produces 5-methylcytosine (5-mc), is an extensively characterized mechanism for epigenetic gene regulation. Neoplastic cells may simultaneously harbor widespread (global) genomic hypomethylation and regional areas of hypermethylation. Although the precise roles of these components are unclear, each component of this "methylation imbalance" may fundamentally contribute to tumor progression. One of the first alterations of DNA methylation to be recognized in neoplastic cells was a decrease in nonspecific (or global) DNA methylation. Recent attention has been directed towards investigating the role of regional hypermethylation of tumor suppressor genes [58]. Despite the frequently observed cancer-associated increases of regional hypermethylation, the prevalence of nonspecific (or global) DNA hypomethylation in many types of human cancer [59,60] suggests that such hypomethylation plays a significant and fundamental role in tumorigenesis. The most likely mechanisms through which nonspecific (or global) DNA hypomethylation may induce neoplastic transformation, *i.e.*, inducing genomic instability [61,62], causing abnormal chromosomal structures [63], and activating oncogenes [64] are biologically plausible.

Two polymorphisms in the MTHFR gene have been reported in most studies: (i) C→T at nucleotide 677, leading to an alanine to valine conversion in the protein [65]; and (ii) A→C in exon 7, causing an alanine to glutamate protein change [66]. The modulation of cancer risk associated with the MTHFR polymorphism is likely be due to differential partitioning of 1-carbon groups between the methylation reactions and DNA synthesis. These risk modifications are however complicated by the fact that global DNA hypomethylation is a risk factor for cancer and hypermethylation of tumor suppressor genes is also a risk factor for cancer. A shift in one carbon units towards DNA synthesis as a result of a polymorphism in MTHFR gene may increase cancer risk by global reductions in DNA methylation, but could be protective by a reduction in methylation in tumor suppressor genes and by decreasing DNA damage. Therefore, the direction of MTHFR polymorphic effect may depend on the relative importance of these mechanisms for specific cancers and the effect of MTHFR polymorphisms on cancer and pre-cancer susceptibility remains highly controversial. A protective effect of this polymorphism was shown for colorectal cancer [67-70] while an increased risk has been reported for endometrial cancer [71] and breast/ovarian cancer [72]. The associations between MTHFR polymorphism and CIN and invasive cervical cancer have also been inconsistent. Two studies have reported increased risk for CIN [73,74] while others have reported no effect of MTHFR polymorphism on CIN [75,76]. Association between MTHFR polymorphism and risk of cervical cancer has also been inconsistent; no association decreased risk or increased risk [77,78]. The colorectal cancer studies suggested that the MTHFR polymorphism reduces colon cancer risk, perhaps by increasing 5, 10 methylenetetrahydrofolate levels for DNA synthesis, but that low folate intake or high alcohol intake may negate some of the protective effect. In a study conducted after folate fortification was implemented in the USA, we reported that MTHFR polymorphism is associated with reduced risk of cervical intraepithelial neoplasia (CIN) 2 or 3 [79]. This protective effect is also likely to be due to a shift in the folate pathway toward DNA synthesis. The human evidence in support of mechanisms involved in these modified risks is limited [80,81].

The inconsistency in associations between MTHFR and pre cancer and cancer could be due to several factors. The association between MTHFR and cancer risk is thought to be modified by folate status; reduced colon cancer risk, but low folate intake may negate some of the protective effect [82]. Not all studies have assessed folate status in relation to MTHFR. Improper control for HR-HPV could also modify these associations. Also, other micronutrients involved in the folate pathway could modify the associations between MHTFR and cancer risk. For example, the activity of MTHFR can be reduced by a low concentration of its cofactor flavin adenine dinucleotide (FAD) or of riboflavin, the precursor of FAD [83] and the association observed between MTHFR and cancer or pre cancer is likely to be modified by the riboflavin status. In vitro studies have shown that polymorphic MTHFR is ~10 times as likely as the wild-type enzyme to dissociate from its FAD prosthetic group and thus become inactivated, whereas high riboflavin status reduces the degree of this dissociation [84]. The other polymorphisms of the folate pathway enzymes, namely methionine synthase (MTR), methionine synthase reductase (MTRR), thymidylate synthase (TS) and cystathionine β-synthase (CBS) may also modify the risk associated with individual risks associated with these polymorphisms. There has been little investigation of the effects of combinations of polymorphisms in the folate pathway.

More studies are also needed to investigate the relations between folate pathway polymorphisms and blood levels of folate and related biomarkers of methylation and DNA damage. Studies conducted so far have been small, with limited statistical power and only investigated only investigated a limited number of polymorphisms and/or biomarkers. A potential difficulty in interpretation of these studies is that any observed difference in biomarker levels by genotype may not be due to the polymorphism under study but to the presence of another polymorphism. Equally, a failure to ob-

serve differences in biomarkers by a specific genotype could be due to the presence of another polymorphism in the same pathway with opposing functional effects. It is important that these studies are conducted in non-diseased individuals. In subjects with decease conditions, it is possible that the condition or its treatment, rather than the underlying genotype, influences biomarker levels and folate nutritional status. MTHFR 677TT genotype is shown to be associated with global DNA hypomethylation in peripheral blood mononuclear cell DNA from largely a deceased (coronary atherosclerosis) polulation [85]. A similar study conducted in healthy individuals, however, reported that lymphocyte DNA stability biomarkers (strand breaks, misincorporated uracil, and global DNA methylation) were similar for all MTHFR C677T or A1298C variants [86]. A recent study demonstrated greater thymidylate synthesis in healthy TT subjects after infusion of ^{13}C-labeled 1-carbon precursors [87], suggesting a shift in the pathway towards DNA synthesis. A few studies have investigated MTHFR and uracil misincorporation, DNA strand breaks, or genetic instability in vivo and in vitro and reported inconclusive results [88-90]. Very few studies have investigated promoter hypermethylation in relation to folate status or folate pathway enzyme polymorphisms. Global DNA hypomethylation has been observed in association with localized hypermethylation of tumor suppressor genes [91]. In contrast, Kang et al. reported that promoter hypermethylation of DNA repair gene O^6-methylguanine-DNA methyltransferase (MGMT) was significantly decreased in subjects who are polymorphic for the MTHFR 677 CT genotype [92]. This is an area which needs muck more work in order to understand cancer risk modification my folate and pathway polymorphisms.

8. SUMMARY AND FUTURE DIRECTIONS

Recent studies that are conducted with proper control for HR-HPV have demonstrated that folate is an important micronutrient in cervical cancer prevention via its influence on HR-HPV. Future studies are warranted to assess whether folate-related biomarkers may be used to identify subjects who are at risk of developing cervical cancer. Currently, there are no validated diagnostic or prognostic criteria that will identify those low-grade precursor lesions (CIN 1) that are destined toward CIN 2 and 3 or cervical cancer. We believe that the conventional histopathological examination of CIN 1 lesions or testing for HR-HPV at a routine care visit provides insufficient information to predict the development of high-grade cervical lesions. We hypothesize that folate-related biomarkers in these lesions and/or alterations in cellular and systemic levels of folate may provide valuable information in this regard. The alterations in these markers are important epigenetic changes 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. Further studies are also needed to systematically evaluate the interactions between folate and other micronutrients and polymorphisms in folate pathway enzymes.

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