Content of folic acid and free homocysteine in blood serum of human papillomavirus-infected women with cervical dysplasia

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Summary

The authors estimated the concentrations of folic acid and free homocysteine in the blood serum of women with CIN III (cervical intraepithelial neoplasia-Burghard's classification) infected with DNA HPV (human papillomaviruses) of type 16 and/or 18. The control group consisted of 49 patients with normal cytological smears without HPV infection. Types 16 and/or 18 DNA HPV were found in 50 patients. This women qualified for the studied group. The sequence of DNA HPV type 16 and/or 18 was identified with the PCR method (polymerase chain reaction). The high-performance liquid chromatography (HPLC) method was employed to evaluate the levels of folic acid and free homocysteine in the blood serum of the examined patients. Significantly lower levels of folic acid and higher levels of free homocysteine were observed in the blood serum of HPV-positive patients with CIN III. The correlation was found between serum concentrations of folic acid and free homocysteine in both groups.

Key words: Tonic acid; Homocysteine; HPV; Cervical dysplasia.

Introduction

The development of cervical neoplasia is a very complex process involving many stages. Human papillomaviruses (HPV) showing tropism towards cervical epithelium play a major role in cervical carcinogenesis. Experimental data has demonstrated that one of the main mechanisms of HPV's molecular activity is the influence of E6 and E7 proteins encoded by high oncogenic risk HPV on p53 and p53 proteins as well as related cell cycle proteins - p107 and p130. E6 inhibits p53 activity [1] while E7 binds to RB gene products (pRB) and by affecting Rb proteins leads to the breaking up of Rb/E2F, which increases the transcriptive concentration of the E2F factor in the cell [2]. Papilloma infection always results in cell cycle regulation changes leading to the loss of cell's control over its proliferation [3]. The previous studies of the nutritional status and CIN (cervical intraepithelial neoplasia) have shown an increased risk to CIN with low dietary intake and low plasma levels of folate, beta-carotene and ascorbate [4-7]. Risk factors for developing CIN and cervical cancer are known to be infection with HPV, non-compliance with Pap smear screening, high parity, lower socio-economic status, smoking, oral contraceptive use, sexual history, and low dietary intake and low tissue levels of folic acid, beta-carotene, alpha-tocopherol and ascorbate [8-11]. In studies that investigated the relationship between HPV infection and nutritional status, low RBC folate was associated with a five-fold higher risk of being HPV positive [12-15], suggesting that folic acid may be a nutritional cofactor responsible for the development of CIN.

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In order to achieve malignant activation of the cervical epithelial cell, HPV must be present along with cofactors at levels sufficient to interfere with cellular control factors. First, HPV must be integrated into the host DNA, which preferentially occurs at a fragile site on DNA [16]. Secondly, activation of specific oncogenes appears to be a prerequisite for HPV-related carcinogenesis to occur [16]. Low tissue folic acid and oxidant stress (low antioxidant nutrients + smoking/ infection) act through different mechanisms to: 1) increase the frequency of fragile sites on DNA [17, 18]; 2) increase the risk for DNA to be attacked by viruses and sustain carcinogenesis [18-21]; as well as 3) decrease the DNA repair and immune function [22] and increase the potential for chromosomal breaks and oncogene expression [23, 24]. The above-mentioned conditions assist an HPV infection in its progress to malignancy.

Several researches have previously hypothesised that a tissue deficiency of folic acid acts as a co-carcinogen in the development of cervical cancer [17, 18, 25]. Since plasma folic acid may not be indicative of local tissue levels, and the method of assessing tissue folic acid concentration had not been previously available, this hypothesis was difficult to test.

Recent data indicate that plasma homocysteine may be a sensitive marker of tissue folic acid availability [26]. Homocysteine, a product of transmethylation, is converted to methionine in a reaction requiring 5-methyltetrahydrofolate as a methyl donor and vitamin B_{12} as a cofactor. Cells retain only small amounts of homocysteine, the majority being released into plasma [27]. Since homocysteine is not supplied with food and folate is acquired in the process of methylation of homocysteine to methionine, it is likely that tissue folate concentrations

are closely associated with the regulation of homocysteine metabolism and the quantity of plasma homocysteine [28]. Oral folic acid administration to healthy adult subjects with low/normal plasma folic acid levels significantly decreases the concentration of homocysteine in plasma, suggesting that folic acid has been limited in various tissues although plasma levels were unable to reflect this [28, 29].

Thus, the aim of this work was to evaluate the level of folic acid and free homocysteine in the blood serum of women with dysplasia associated with HPV infection.

Materials and Methods

The following patients were qualified for the studies: a) 50 patients infected with HPV 16 and/or 18 type of high oncogenic risk and with histopathologically diagnosed CIN III (histological classification was performed according to the international nomenclature of Burghard and Ostor [30]; b) 49 women (a control group) whose cytological smears used for routine gynaecological examination were evaluated as the first or second cytological degree according to the Papanicolaou classification without HPV infection. In these women, gynaecological examinations excluded the presence of any lesions in the genital tract. The women with no history of digestive or urinary system illnesses and without any other infectious diseases were qualified for the study. The age of patients and season in which the examinations were performed were taken into account. The patients with intraepithelial cervical neoplasm III without any lesions outside this area, were of excellent somatic health and their diet was not supplemented with vitamins, nor were they medicated for any other diseases. The patients of the two groups did not differ statistically in terms of age. The average age was 38.2 years for patients with CIN and 34.8 years for those with regular cytological smears. Sixty-seven percent of CIN patients had an elementary education, and only 9% a higher education. This differed significantly from the control group of patients with regular smears, i.e., 54% with an elementary and 20% with a higher education.

Identification of HPV type 16 and 18 of high oncogenic risk in the group of patients was performed in the Department of Molecular Virology, Adam Mickiewicz University in Poznań (Poland) with polymerase chain reaction. PCR was carried with specific sets of primers (Table 1). The final mixture contained 1 μM primer, 200 μM deoxynucleotide triphosphates, 1x PCR buffer (10 mM Tris -HCl, pH: 8.3; 50 mM KCl; 3.5 mM $MgCl_2$), 200 μM deoxynucleotide triphosphates; 1.OU/25 μl of Tag polymerase mixture. The samples were amplified for 30 cycles. Each cycle consisted of the following steps: denaturation at 95°C for one minute, annealing at 59°C for 30 seconds, primer extension at 72°C for one minute in a DNA thermal cycler (Brometra). After amplification the PCR products were analysed by Southern blot hybridization with ^{32}P-labelled probe specific for HPV 16 and 18 [31].

Table 1. — *Primers for PCR study.*

Primers	Region of amplification	Sequence 5'-3'	Product size
MY09 MY11	L1	CGTCCMARRGGAWACTGATC GCMCAAGGWCATAAYAATGG M=A+C,R=A+G,W=A+T, Y=C+T	450 bp
HPV16/L1A HPV16/L1B	L1	GCCTGTGTAGGTGTTGAGGT TGGATTTACTCCAACATTGG	264 bp
HPV18/L1A HPV18/L1B	L1	GTGGACCAGCAAATACAGGA TGCAACGACCACGTGTTGGA	162 bp
HPV18ME12 HPV18ME50	E6	CACGGCGACCCTACAAGCTACCTG TGCAGCACGAATTGGCACTGGCCTC	404 bp

The blood samples were obtained from patients to determine the content of folic acid and free homocysteine in the blood serum. The samples were placed on ice, mixed with EDTA, deproteinized by adding sulfosalicylic acid and centrifuged for 20 minutes. The samples of deproteinized plasma were stored at -70°C until analysis. Folic acid and homocysteine were analysed by the high-performance liquid chromatography (HPLC) method, i.e., reverse-phase HPLC. The chromatograms of folic acid were developed according to the method of Zeitler *et al.* [32] and Cashmore *et al.* [33] on Ultrasphere ODS column (250 x 4.6 mm) in a gradient (0-15%) of acetonitrile in 0.1 M acetate buffer, pH 5.5 at a flow of 1.5 ml/min and detection at 245 nm. A folic acid preparation - Pteroglutamic acid (Pte Glu 1) from Sigma Chemical was used as a standard. The analysis of free plasma homocysteine was carried out using the method of Araki and Sako [34]. HPLC was performed with Beckman chromatograph (Gold System 126/166) with analytical and quard columns. The fluorescent intensity was measured at 550 nm using a Beckman fluorescent spectrophotometer. DL-homocysteine (Sigma) was used as a standard.

The results were statistically analysed and summarised in correlational tables, calculating the structural indexes, arithmetic means and mean errors for measurable features. A parametric test was used as a method of statistical measurement, including the Student's t-test and F test of variance analysis. Statistical significance was found at $p < 0.05$. Correlations between free homocysteine and folic acid concentrations were estimated by establishing a correlation coefficient.

Results

The mean folic acid content in the blood serum of patients with CIN III associated with HPV infection type 16 or/and 18 was statistically lower (17.225 nmol/l) than in controls (24.341 nmol/l) (Table 2). The comparison of reliability ranges demonstrated that the range from 1.7232 to 9.3196 is the most important one for the difference between the mean values. Because the range does not include the 0.0 value we can be 95% certain that there is a statistically significant difference between the mean values. The comparison of values using the Student's t test ($t = -2.832$; $p < 0.01$) and analysis of the F variation ($F = 1.064$, $p = 0.0938$) revealed statistically significant differences between the mean values. As can be deduced from descriptive statistics, the concentration of folic acid in the control group undergoes normal decomposition. The diagonal distribution of folic acid concentration in the study group is similar to normal, but kurtosis is smaller than -2.0 and thus the distribution is more flattened.

The mean free homocysteine content in blood sera of patients with CIN III associated with HPV infection type 16 and/or 18 (1.382 μmol/l) was statistically significantly higher than in controls (1.152 μmol/l) (Table 3). The

Table 2. — *Folic acid levels in blood sera of women with cervical dysplasia and HPV infection* [a,b].

Histopathology/Cytology	Arithmetic mean	Mean error	95% CI	Standardised Skewness	Standardised Kurtosis	p value[c]	n
I°+II° (control) HPV negative	24.341	5.821	21.478-25.967	0.575 p = 0.489	-1.051 p = 0.293		49
CIN III HPV positive	17.225	8.241	14.674-21.668	0.336 p = 0.718	-3.571 p = 0.0002	< 0.01	50

a: folic acid levels in μmol/l.
b: Abbreviations are as follows: HPV, *human papillomavirus*; CIN III, cervical intraepithelial neoplasia III°, histopathological degree according to Burghard's classification, I° and II°, 1st and 2nd cytological degree according to Papanicolaou classification; n, no. of patients 95%CI, 95% confidence interval.
c: In relation to the control group; Student't t- test: = -2.832, and F = 1.064, p = 0.0938, p<0.01.

Table 3. — *Free plasma homocysteine levels in blood sera of women with cervical dysplasia and HPV Infection*[a,b].

Histopathology/Cytology	Arithmetic mean	Mean error	95% CI	Standardised Skewness	Standardised Kurtosis	p value[c]	n
I°+II° (control) HPV negative	1.152	0.112	1.138-1.226	0.854 p = 0.377	-0.912 p = 0.351		49
CIN III HPV positive	1.382	0.114	1.314-1.384	0.764 p = 0.423	0.952 p = 0.324	< 0.001	50

a: free homocysteine levels in μmol/l .
b: Abbreviations are as follows: HPV, *human papillomavirus*; CIN III, cervical intraepithelial neoplasia III°, histopathological degree according to Burghard's classification , I° and II°, 1st and 2nd cytological degree according to Papanicolaou classification; n, no. of patients 95% CI, 95% confidence interval.
c: In relation to the control group; Student't t test: = -5.348, and F = 1.054, p = 0.871, p < 0.001.

comparison of reliability ranges demonstrated that the range from -0.0223 to -0.1034 is the most important for the difference between the mean values. Since the range does not include the 0.0 value we can be 95% certain that there is a statistically significant difference between the mean values. The comparison of values using the Student's T-test ($t = -5.348$, $p < 0.001$) and analysis of the F variation ($F = 1.054$, $p = 0.871$) revealed statistically significant differences between the mean values.

The coefficient of correlation between the concentration of free homocysteine and folic acid in the control group was: $r = +0.267049$ and $p < 0.001$, and in the study group $r = +0.342423$ and $p < 0.001$. A correlation was found between serum concentrations of folic acid and free homocysteine in both groups.

Discussion

Our earlier studies [10, 14, 15] also point to a decreased level of blood serum antioxidant and folic acid in women with cervical dysplasia, which is more distinct in cases of concomitant HPV infection. This fact made us try to elucidate these dependencies. Accordingly, all factors which could affect the antioxidant level in blood sera were consistently eliminated. The same conditions of taking blood samples and their storage were maintained, and only those women were qualified for studies whose anamneses eliminated illnesses of the alimentary tract and urinary system as well as infectious diseases. Both the age of the patients and the season of the year in which the studies were carried out were taken into consideration. The patients with cervical intraepithelial neoplasia, except for changes in this region, were in good physical health. Thus, the level of folic acid should not be suspected to result from absorption disturbances due to other inflammatory non-sexual or neoplastic diseases.

Many experimental, clinical and epidemiological data indicate that folate deficiency can increase cell susceptibility to neoplastic transformations [35, 36]. Folic acid deficiency increases the number of cells arrested in the S phase [37], which exhibit higher sensitivity to viral infection. It leads to point mutations [36] which often cause shifts in genetic code reading [37] as well as chromosome breaks [38].

Folic acid deficiency is also the cause of hypomethylation (decrease in the level of DNA methylation accompanied by S-adenosylomethionine) [39], which by decreasing stabilisation of the helix structure, leads to decreased expression of viral genes (thymidine kinasis HSV1) and/or protooncogenesis [40, 41] and megaloblastic anaemia. Pietrantoni *et al.* [42] studied the regulation of HPV oncogene expression by folic acid. They studied c-fos, c-jun and HPV E6 expression in Caski (HPV 16-positive) cell lines treated with folic acid. They found diminished c-fos and c-jun expression using Western blot when folate concentrations of more than 100 nM were used. Similarly, E6 protein expression was diminished at concentrations of more than 10 nM, suggesting that the mechanism by which the transcription regulators - c-fos and c-jun were controlled was diminished by viral E6 expression.

The mechanism through which the local folate deficiency can contribute to an increased risk of malignant changes remains unclear. However, it should be noted that folic acid plays a role in thymidine acid synthesis by activating it [36, 37]. It has been shown that thymidine deficiency causes changes in the nucleotide pool [43, 44] and influences such phenomena as decreased DNA replication [43], decreased pool of deoxynucleotides and excessive incorporation of uracil into DNA. Repair of this incorrectly incorporated uracil in the presence of folate deficiency results in so-called "catastrophic repair" during which further incorporation of uracil can occur [43, 44, 45]. It seems that this disturbance of deoxynucleotide biosynthesis is responsible for the carcinogenic effects of folate deficiency [37].

Rogers and Newberne's studies [43] showed that a diet poor in methionine, choline, foliates and vitamin B12 promotes chemical carcinogenesis in rats. This carcinogenic effect is probably connected with a decrease in S-adenosinomethionine concentration resulting from dietary deficiency in methyl groups. [35, 39]. S-adenosinomethionine is a methyl group donor in many biochemical reactions including DNA methylation [37]. Although the role of DNA methylation is not fully understood, many studies have indicated that the fall of methylation is related to gene activation [41]. Some studies [25, 44] suggest that DNA hypomethylation as well as specific proto-oncogene hypomethylation plays an important role in carcinogenic process initiation .

Experimental studies [45] showed that moderate folate deficiency, induced by a diet leading to deficiency that does not stop growth or cause anaemia , intensifies neoplastic processes, especially when it coincides with a predisposition to the development of neoplasia.

Clinical studies attempting to relate folic acid deficiency with dysplastic lesions of cervical [12, 14, 15, 46, 47], bronchial [48] and colonic [49] epithelium suggest quite close relationships.

Orr *et al.* [46] demonstrated decreased levels of folic acid in 30.8% of patients with a high dysplasia degree and a statistically significant decrease depending on the degree of dysplastic lesions of the cervix. Similar results were found in Liu *et al's.* studies [50] where a relative risk of dysplasia, standardised to other risk factors including the presence of HPV infection, was 1.4 to 1.9 depending on the serum folic acid level. Butterworth's studies [12, 13] as well as the results presented in our study confirm the observations described above. However, the results of Potischman *et al.* [51], evaluating the serum level of folates in women with cervical neoplasia compared to controls, did not show statistically significant differences in the populations studied.

According to our present knowledge, folic acid deficiency is the main reason for hyperhomocysteinemia. The blood homocysteine content is mainly the subject of studies concerning its influence on atheromatous intensification and increased risk of ischaemic heart disease and peripheral vessel thrombosis [52, 53]. Less frequently it is the subject of studies on carcinogenesis and, if so, it is strictly connected with the problem of the content of folates. The experimental studies on animals and clinical studies on humans indicate that there is an increased level of homocysteine in blood as a result of dietary folate deficiency and its damaging effects on blood vessel walls. The pathological mechanisms of homocysteine activity in atheromatous processes have not been thoroughly examined yet. An excessive amount of this amino acid can damage endothelial cells, decrease relaxing factor production, stimulate smooth muscles cell proliferation and cause blood clotting disorders. At present the clinical data are sufficient to demonstrate a relationship between blood homocysteine content and the development of atheromatosis. Folates are likely to have the strongest effects on blood homocysteine content in healthy people.

The evaluation of homocysteine content in the blood serum of patients with breast cancer treated with tamoxifen showed a decrease in hyperhomocysteinemia from 29.8% to 24.5% after a 13-18 month-long treatment [54]. A reduction of cardiovascular mortality was found in patients on adjuvant therapy with tamoxifen [54]. Cole *et al's.* study [55], evaluating the correlation between total homocysteine and cyclosporine concentrations in cardiac transplant recipients, suggests that folate and renal glomerular dysfunctions are important contributory factors; however, whole blood cyclosporine concentrations may also predict the degree of hyperhomocysteinemia in this population.

The studies concerning smoking patients with pancreatic cancer showed decreased folate and increased homocysteine levels with higher pancreatic cancer risk. The results support the hypothesis that maintaining an adequate folate status may reduce the risk of pancreatic cancer [56]. Moreover, experimental studies on rats concerning the development of macroscopic colonic neoplasm conducted by Kim *et al.* [57] indicated that a) increasing dietary folate level four times above basal requirements led to a progressive reduction in the evolution of macroscopic neoplasms from microscopic foci, and b) folate supplementation four times exceeding requirements did not bring further benefits. Giavannuci's clinical studies showed a reverse correlation between folic acid consumption and colon polyps [58].

Our own studies confirm the observations of other authors about the statistically significant lower folic acid and higher free homocysteine concentrations in the blood serum of women with carcinogenic processes in the cervix (CIN HPV +). Correlations between the concentration of folic acid and free homocysteine in the study group of CIN HPV (+) patients and control group suggests that folate and cervix dysplasia associated with HPV are important contibutory factors.

Conclusions

In conclusion the present study demonstrates that folic acid may play protective role in the carcinogenesis colli uteri associated with HPV infection.

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