Elevated Homocysteine, an Emerging Biomarker for Cervical Cancer, A Case-Controlled Study

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Abstract: *Background*: Elevated homocysteine, an emerging biomarker for cervical cancer is amenable to treatment by cheap and effective nutritional interventions.

Methods: From April 2012 through September 2012, a case-controlled study was performed in the Gynae oncology unit of Bangabandhu Sheikh Mujib Medical University (BSMMU) and the National Institute of Cancer Research and Hospital (NICR & H), in Dhaka, Bangladesh. The objectives were to measure the serum homocysteine levels in women with squamous cell carcinoma of the cervix and in normal controls. Fifty women with invasive squamous cell carcinoma and 50 normal controls were compared for demographic and socioeconomic differences. Blood was tested for homocysteine levels.

Results: Among cases of cervical cancer, 82% had homocysteine level between 4.5-15 µmol/L & 14% had high homocysteine level (>15 µmol/L). Whereas in control group, 98% had homocysteine level between 4.5-15 µmol/L and 2% had homocysteine <4.5 µmol/L (low). High level of homocysteine (>15 µmol/L) was not observed in any patient in the control group. The mean homocysteine level in cervical cancer patients was also higher (10.88 µmol/L) than that of controls (8.50 µmol/L) and this difference was statistically significant.

Histological grade and clinical stage of cervical cancer did not correlate with serum homocysteine level.

Conclusion: There is a difference in homocysteine levels in women with cervical cancer. Homocysteine, which is associated with nutritional deficiencies of fruits and vegetables, may play a role as a marker for or as a co-factor in malignant transformation of cervical squamous epithelium. Larger studies are needed to further study this association. However public awareness is important regarding the role of fresh vegetables and fruits containing vitamin B (folate-vitamin $B_{9;}$ vitamin $B_{12;}$ vitamin B_6) to reduce homocysteine and its possible consequences of cancer prevention.

Keywords: Biomarker, cervical cancer, homocysteine.

INTRODUCTION

The uterine cervix is the commonest site for cancer of the female genital tract. In Low and Middle Income Countries (LMICs), cervical cancer is one of the leading cancers among women and is the second most common cause of cancer related morbidity and mortality [1]. In Bangladesh, carcinoma cervix is the second common cancer among women after breast cancer. The true incidence of cervical cancer is unknown in the country; 13000 new cases and 6000 cancer deaths per year have reported from limited academic registries (IARC 2002). Considering the disease burden and its consequences, it has become essential to identify a marker for cervical cancer for the benefit of the patients.

Cervical intraepithelial neoplasia (CIN) or cervical dysplasia, is a pre-malignant condition which can progress to cervical cancer. A progression of epithelial cell changes across a continuum of lesions classified as (CIN) I, II, III and carcinoma in situ (CIS) are the precursor lesions for invasive cervical cancer [2].

Pre-malignant and malignant disorders of cervix are caused by some oncogenic strains of Human Papilloma Virus (HPV) which are transmitted sexually [3]. In about 70-90% of women infected by HPV, the virus is cleared of the body by the natural immune system. CIN develops in about 28% of women infected by HPV and small proportions of unfortunate women develop cervical cancer [4]. Multiple host factors like early sexual activity, multiple sexual partners, sexually transmitted diseases, Oral Contraceptive Pill (OCP) use, smoking, low socioeconomic status, poor diet and immunosuppression are involved in development and progression of CIN and cervical cancer.

Deficiency of several nutrients allow persistence of HPV infection which influence progression of early precancerous lesions to invasive cancer [5]. Treatment

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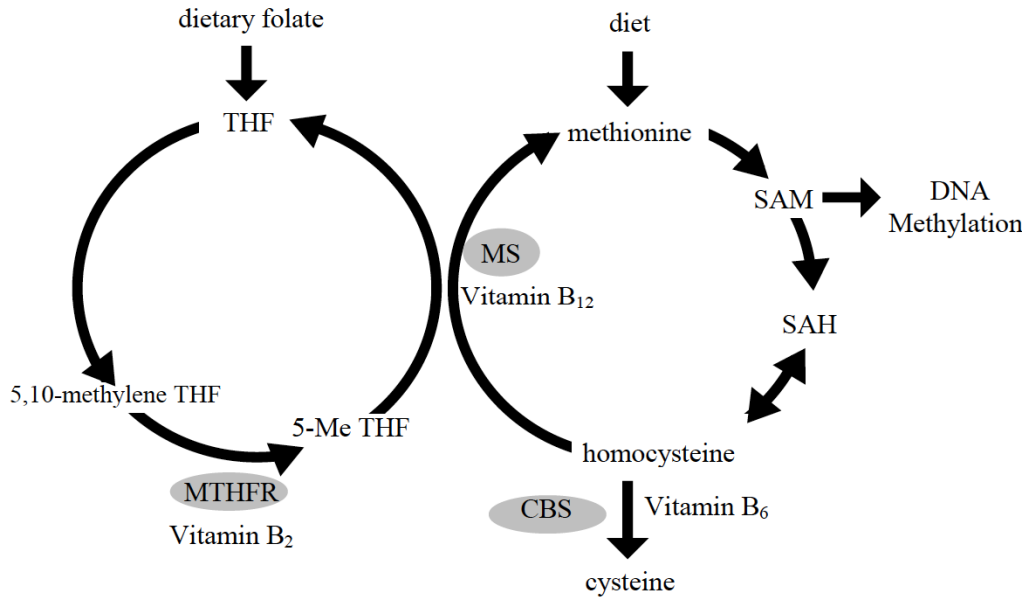

Figure 1: Metabolism of homocysteine. Folate is converted to 5,10-methylene tetrahydrofolate (5,10-methylene THF) which is reduced to 5-methyl tetrahydrofolate (5-Me THF) catalyzed by methylene tetrahydrofolate reductase (MTHFR). Homocysteine captures a methyl group from 5-Me THF to form methionine, catalyzed by vitamin B_{12} dependent methionine synthase (MS). Methionine is converted to ***S***-adenosyl methionine (SAM) which donate methyl group and causes DNA methylation. Homocysteine is catabolized to form cysteine by vitamin B_6 dependent cystathionine β synthase (CBS).

of these nutritional deficiencies is associated with regression of dysplastic lesion [3].

Homocysteine is a sulphur-containing amino-acid present in all cells of the body. Metabolism of homocysteine occurs in one-carbon metabolic pathway. In this pathway, in presence of vitamin B_{12}, homocysteine is converted to methionine. Methionine is needed for DNA synthesis, repair and methylation. Impairment of homocysteine metabolism due to low vitamin B results in accumulation of homocysteine and deprivation of methionine. Lac of methionine leads to DNA instability & genetic damage. HPV integrate into the host genome at or near fragile sites and promote carcinogenesis.

Another reason for elevated homocysteine in cervical cancer is that- during tumor progression, rapid proliferation of tumor cells causes folate depletion to meet the demand of increased protein synthesis in malignant cells. So homocysteine is not converted to methionine. Hypomethylation in certain genes in cervical cancer causes selective growth advantage. Elevated levels of homocysteine is found in rapidly proliferating tumor cell lines and levels decrease as the cells start dying thus establishing homocysteine as proliferation marker.

Some clinico-epidemiological studies done previously in different countries have shown an association between elevated homocysteine with cervical cancer and pre-cancers [5-11]. There are also some studies which have shown that elevated homocysteine level enhances the effects of risk factors (eg. HPV) of cervical cancer and promote cancer development [12].

In Bangladesh where cervical cancer is the most common genital tract cancer, no studies done previously to see the association of elevated homocysteine with cervical cancer risk. If the association between homocysteine and cervical neoplasia can be established, development and progression of cervical pre-cancer and cancer can be prevented by reducing homocysteine level which can be achieved successfully by simple dietary intervention. Dietary counseling regarding increased intake of fresh fruits and vegetables containing vitamin B or vitamin B supplementation can significantly reduce total plasma homocysteine [2].

Scarcity of prevention and screening programs in low resource countries, particularly in rural areas is responsible for high incidence of cervical cancer [13]. As micronutrients in diet can prevent cancer, cancer prevention programs responsive to appropriate diet and vitamin supplementation need special attention.

MATERIALS AND METHODS

From April 2012 through September 2012, a case-controlled study was performed in the Gynae oncology

unit of Bangabandhu Sheikh Mujib Medical University (BSMMU) and the National Institute of Cancer Research and Hospital (NICR & H), in Dhaka, Bangladesh. The objectives were to measure the serum homocysteine levels in women with squamous cell carcinoma of the cervix and in normal controls.

Patients with histopathologically confirmed squamous cell carcinoma of uterine cervix were included as cases. Women of same demographic and socioeconomic parameters with normal cervical examination by screening test "Visual Inspection with Acetic Acid" (VIA) were recruited as control. To have a homogenous result, women with adenocarcinoma, adenosquamous and other rare histologic types of cervical cancer were excluded from the study. Also excluded were women with conditions associated with elevated homocysteine levels such as: pregnant/nursing women, previously treated cervical cancer and pre-cancer, patients receiving chemotherapy for cervical cancer, malignancy of any other organ, women with diabetes, osteoporosis, cardiovascular, neurological and renal disease, women on medication with anticonvulsants, women with BMI >30 kg /m^2, or women with substance abuse disorders such as tobacco, alcohol, or other drug addictions.

As level of homocysteine is influenced by many factors mentioned above, it was possible to include 50 cases of invasive squamous cell carcinoma of cervix and they were matched with 50 controls (with normal cervix) of similar characteristics within the defined period of study (6 months).

Study Procedure

Cases were collected from Gynae-oncology outdoor of BSMMU and NICR & H. Admitted indoor patients waiting for treatment were also included. Women who came to VIA centre of BSMMU for cervical cancer screening and diagnosed VIA-negative (normal cervix) were selected as controls. Detailed information was obtained on demographic characteristics, reproductive history, exogenous hormone use, family history, medical history and personal history of smoking, diet and other lifestyle factor. Findings of relevant physical examinations and necessary investigations were also recorded.

Pelvic examination of cancer patients was done for staging of cancer. In selected cases Examination under Anaesthesia (EUA) was done to confirm the staging.

Blood glucose reports of patients were evaluated to exclude diabetes mellitus as it may influence serum homocysteine level.

Reports of histopathological examination of cervical tissue from cancer patients were recorded properly with mentioning of histological grade of cancer.

After collection of all information, the study subjects were divided into two groups

1) Group A (case): Patients with squamous cell carcinoma of uterine cervix

2) Group B (control): Patients with normal cervix

Laboratory Test

The serum homocysteine estimation was done by AxSYM system based on the Fluorescence Polarization Immuno Assay (FPIA) technology (Abott Diagnostic 2006). Bound homocysteine (oxidized form) is reduced to free homocysteine that is enzymatically converted to S-Adenyl homocysteine (SAH) which is measured for quantitative data.

Data Analysis

Data were collected, compiled and analyzed by using the computer software Statistical Package for the Social Sciences (SPSS) for Windows (version 19.0; SPSS Chicago,IL,USA). The results were expressed as the mean ±SD (Standard deviation). Pearson chi-square test was used for analysis of all categorical variables. Unpaired Student's "t" test was performed for quantitative parameters. P value <0.05 was considered as significant.

Ethical Aspect

Ethical clearance for the study was taken from the Institutional Review Board (IRB) of BSMMU and Ethical Committee of NICR & H, Dhaka.

RESULTS

The age, parity, education, and occupations of patients are summarized in Tables **1** and **2**. Socioeconomic status is summarized in Table **3**. Table **4** shows the Oral Contraceptive Pill (OCP) use and BMI of the subjects.

The patients with cervical cancer were categorized as group A and those with healthy cervix were categorized as group B for detailed statistical analysis. The two study groups were almost identical in terms of

Table 1: Age and Parity Distribution of the Study Subjects

Age (years)	Group A (n=50)		Group B (n=50)		P value
	No.	(%)	No.	(%)	
<35	05(10.0)		10(20.0)		0.29^{ns}
35-55	41(82.0)		38(76.0)		
>55	04(8.0)		02(4.0)		
Mean±SD	46.28±8.39		43.66±8.93		0.13^{ns}
Parity					
0-2	20(40)		27(54)		0.22^{ns}
≥ 3	30(60)		23(46)		

Group A: Case (squamous cell carcinoma of uterine cervix).
Group B: Control.
Unpaired Student's 't' test/ Chi-square test.
ns = Not significant.

Table 2: Education and Occupation of the Study Subjects

Education	Study group				P value
	Group A (n=50)		Group B (n=50)		
	No.	(%)	No.	(%)	
Illiterate	19(38)		06(12)		0.005**
Literate	31(62)		44(88)		
Occupation					
Housewife	42(84)		43(86)		0.77^{ns}
Service	08(16)		07(14)		

Group A: Case (squamous cell carcinoma of uterine cervix).
Group B: Control.
Chi-square test.
** = Significant at P<0.05.
ns = Not significant.

Table 3: Socioeconomic Status of the Study Subjects (by Monthly Income)

Socioeconomic status	Group A (n=50)		Group B (n=50)		P value
	No.	(%)	No.	(%)	
Poor	05(10.0)		05(10.0)		0.863^{ns}
Low	38(76.0)		35(70.0)		
Middle	06(12.0)		08(16.0)		
Upper middle	01(2.0)		02(4.0)		

Group A: Case (squamous cell carcinoma of uterine cervix).
Group B: Control.
Poor: Income ≤Tk. 4,920/month.
Low: Income >Tk. 4,920 - 24,600/month
Middle: Income >Tk. 24,600 – 49,200/month.
Upper middle: Income >Tk. 49,200 – 1,23,000/month.
Source: Pew Research Center Analysis of data from the World Bank.
Chi-square test.
^{ns} = Not Significant at P<0.05.

Table 4: Comparison of OCP Use and BMI between Study Groups

OCP	Group A (n=50)		Group B (n=50)		P value
	No.	(%)	No.	(%)	
Yes	27(54)		33(66)		0.22^{ns}
No	23(46)		17(34)		
BMI (kg/m^2)					
Mean±SD	21.30±1.72		21.78±1.85		0.182^{ns}
Range	19.00-26.00		19.00-27.00		

Group A: Case (squamous cell carcinoma of uterine cervix).
Group B: Control.
Chi-square test/Unpaired Student's 't' test.
ns = Not significant.

age, parity, occupation, monthly income, OCP use and BMI, which are the potential confounders that may affect the risk for cervical cancer as well as serum homocysteine level. But significant difference was observed in education level.

In the present study maximum number of women in both group A and B were housewives, 84% and 86% respectively. Regarding education, 38% patients with cervical cancer were illiterate. 88% of patients without cervical cancer (Group-B) were literate & only 12% were illiterate. Cases in the study were significantly less educated than controls.

Regarding socioeconomic status (by monthly income), 76% of cases of cervical cancer and 70% of controls (without cervical cancer) were from low income group. Both the study groups are not different significantly in this respect.

Table **5** identifies the homocysteine levels of women with cervical cancer versus controls.

Tables **6** and **7** analyzed homocysteine levels by grade and stage of cancer.

82% of patients with invasive cervical cancer had homocysteine level between 4.5 - 15 µmol/L and 14% had homocysteine level >15 µmol/L. In contrast, 98% of non-cases had homocysteine level between 4.5-15 µmol/L and none was found to have homocysteine level >15 µmol/L. This result indicated significantly higher level of homocysteine amongst patients with cervical cancer. Moreover, in this study the mean homocysteine level in cervical cancer patients was higher (10.88 µmol/L) than that of controls (8.50 µmol/L) and this difference was statistically significant.

Histological grade and clinical stage of cervical cancer did not correlate with serum homocysteine level. Mean homocysteine concentration was lower (10.22 µmol/L) in grade I, well-differentiated cancer than grade II & grade III cancer jointly (11.09 µmol/L) but the difference was not statistically significant. No statistically significant difference in homocysteine level was observed between early stage (stage IA, lB, IIA) and advanced stage (Stage IIB, IIIA, IIIB) of cancer in this study although mean homocysteine level (12.05 µmol/L) was higher in advanced stage than those in early stage (10.10 µmol/L).

Table 5: Serum Homocysteine Levels of the Study Subject

Serum (µmol/L)	Group A (n=50)		Group B (n=50)		P value homocysteine
	No.	(%)	No.	(%)	
<4.5 (low)	02 (4.0)		01 (2.0)		0.01*
4.5-15 (medium)	41 (82.0)		49 (98.0)		
>15 (high)	7 (14.0)		00		
Mean±SD	10.88±4.51		8.50±2.57		0.002**

Group A: Case (squamous cell carcinoma of uterine cervix).
Group B: Control.
Chi-square test/Unpaired Student's 't' test.
* = Significant at P<0.05.
** = Significant at P<0.01.

Table 6: Effect of Histological Grade of Carcinoma Cervix on Serum Homocysteine Level

Histological grade	(n=50)	Homocysteine level	P value
		Mean ±SD	
Well-differentiated (Grade-I)	12	10.22 ±2.61	0.57^{ns}
Moderate to undifferentiated (Grade II and III)	38	11.09 ±4.97	

Group A: Case (squamous cell carcinoma of uterine cervix).
Unpaired Student's 't' test.
ns = Not significant.

Table 7: Effect of Carcinoma Stage on Serum Homocysteine Level

Carcinoma stage	(n=50)	Homocysteine level	P value
		Mean ±SD	
Early stage (Stage IA, IB and IIA)	30	10.10 ±3.87	0.13^{ns}
Advanced stage (Stage IIB, IIIA and IIIB)	20	12.05 ±5.20	

Group A: Case (squamous cell carcinoma of uterine cervix).
Unpaired Student's 't' test.
ns = Not significant.

DISCUSSION

Maximum number of women in both the groups in this study belonged to age group 35 to 55 years. Age incidence of invasive cancer of the cervix has two peaks, one at about 35 years and another at about 50-55 years [14]. The Third National Health and Nutrition Examination Survey in Americans showed that total homocysteine concentration increased with increasing age but participation bias is unlikely in this study as the distribution of cases was not significantly different from that of the controls on age.

Women with high parity have increased risk for squamous cell cervical cancer than women who have 0 to 2 babies. In this study 60% of cervical cancer cases had parity ≥3 in comparison to controls (46%). In a study by Yilmaz N *et al*. (2006) revealed that the homocysteine levels were positively associated with the number of deliveries [15]. In the present study, cases were not significantly different from control with respect to parity, which eliminate the effect of this confounder on the association of homocysteine level and cervical cancer risk.

Carcinoma cervix is more prevalent amongst women with a low income and indifference education [14]. In this study most of the cases (76%) were from low income group and they were compared with healthy controls of similar socioeconomic status.

OCP is a known risk factor for cervical cancer. There is also association between serum homocysteine and OCP use. The possible confounding effect of OCP use on the homocysteine level was not observed in this study. 66% of women in control group and 54% carcinoma cervix cases used OCP and the difference was not statistically significant. Serum homocysteine increases with increased BMI. In the present study cervical cancer patients and their matched controls were almost similar with mean BMI of 21.30 kg/m^2 and 21.78 kg/m^2 respectively.

For analysis of serum homocysteine levels of the study subjects, the 5^{th}-95^{th} percentile range of homocysteine, 4.5-15.1 µmol/L was used which is similar to that reported in the Third National Health and Nutrition Examination Survey [16]. In the present study, 14% of patients with invasive cervical cancer had homocysteine level >15 µmol/L (high). In contrast, none of the controls had homocysteine level >15 µmol/L. This result indicated significantly higher level of homocysteine amongst patients with cervical cancer. Moreover, in this study the mean homocysteine level in cervical cancer patients was higher (10.88 µmol/L) than that of controls (8.50 µmol/L) and this difference was statistically significant inferring that homocysteine levels are significantly associated with cervical cancer.

Among four previous studies examining the association between serum homocysteine and invasive

cervical cancer, three showed a significant increase in cancer risk with elevated plasma homocysteine [6-8]. Weinstein *et al*. (2001) and Ziegler *et al*. (2002) showed a significant increase in the risk of cervical cancer for women with elevated plasma homocysteine [6,7]. In India a study conducted by Kohaar *et al*. (2010) established an association of elevated homocysteine level with cervical cancer risk [8]. The present study supports the findings of these studies. The fourth study was a small study which included 26 in-situ (CIS) and 13 invasive cervical cancer cases. This study also showed positive association of serum homocysteine with increased risk of cervical cancer but result was not statistically significant [17].

Five studies to examine the association of serum homocysteine and cervical pre-cancers revealed that three studies were able to show significant association [8,10,11]. Thomson *et al*. suggested serum homocysteine as a risk factor for cervical dysplasia [10]. Kwasniewska *et al*. (2002) showed that serum homocysteine is significantly higher in HPV positive patients with CIN III [11]. One study by Goodman *et al*. (2000) found a positive trend in the odds ratio for cervical pre-cancers for increased plasma homocysteine [9]. Another study by Sedjo *et al*. (2003) did not support a role of homocysteine in cervical pre-cancers [18]. In the last study, the sample size was small which limited the power of the study.

In the present study, influence of histological grade and clinical stage of cervical cancer on serum homocysteine level was examined. No statistically significant difference in homocysteine level was observed between different grades and stages. These findings were similar to a study done by Weinstein *et al*. and indicated that effect of disease progression did not affect the homocysteine level.

Until more interventional studies have been completed, it is reasonable to assume nutrient supplementation to individual at risk of cervical cancer. This action will reduce homocysteine level and prevent development and progression of cervical cancer [3]. Cancer prevention and detection efforts will be more beneficial for low resource countries as it can reduce future disease burden and save resources necessary for other development programs of the country.

There are several limitations to this study. This was a small sample size and statistically underpowered. HPV–DNA test was not done in this study. Some authors of different studies argued that HPV-DNA test should be done for precise interpretation of findings, it is revealed that studies taking HPV infection into account do not differ significantly from those studies that did not control for it. The levels of serum folate and vitamin B_{12} was not measured in this study to strengthen the fact that deficiencies of these vitamins are associated with elevated homocysteine level. Very few studies have examined the association of homocysteine and cervical cancer risk. So comparison of the present study with other recently published similar studies was scarce. The uncertainity is: elevated homocysteine is found to be associated with cervical cancer risk and can be used as a marker of cervical cancer but elevated homocysteine is not specific for cervical cancer. It is also involved in cancer of other organs of the body and may act as risk factor for atherosclerosis, cardiovascular disease, peripheral vascular disease, stroke, diabetes, osteoporosis, dementia and Alzheimer's disease.

CONCLUSION

The present study was able to establish the association between elevated serum homocysteine level and invasive cervical cancer. Further studies with large sample size and enough power to detect small effect is needed to establish homocysteine as marker of cervical cancer.

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