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PLASMA HOMOCYSTEINE, FOLATE AND VITAMIN B_{12} LEVELS IN PATIENTS WITH LUNG CANCER

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***Aim*: Disorders in the metabolism of homocysteine and B vitamins, which are involved in a one-carbon transfer reaction and important for DNA synthesis and methylation, have been hypothesized to be associated with carcinogenesis. The purpose of this study is to evaluate the levels of homocysteine, vitamin B_{12} and folic acid in patients with newly diagnosed lung cancer and determines whether they might be used as an accurate tumor marker for monitoring the patients if they are found to be elevated in lung cancer. *Materials and Methods*: Forty male patients with lung cancer were included in this study. Age-matched forty healthy males who had not malignant disease or had not received any drug affecting plasma homocysteine levels were selected as control group. Homocysteine, vitamin B_{12} and folate levels were measured in the samples obtained from the patients and controls. *Results*: Mean age of the patients with lung cancer was 58.7 ± 9.9 years. All the patients were cigarettes smokers. Mean daily consumption of cigarettes was 2.0±0.7 packs and mean duration of smoking was 30 ± 11 years. Histologic type of carcinoma was found to be squamous cell carcinoma in 55%, adenocarcinoma — in 35%, and small cell carcinoma — in 10% of the cases. Clinical stage was stage IA in 20%, stage IB — in 20%, stage IIA — in 2.5%, stage IIB — in 10%, stage IIIA — in 12.5%, stage IIIB — in 20%, and stage IV — in 15% of the cases. Mean homocysteine level was 15.3 ± 7.3 μmol/l in the patients with lung cancer while 9.8 ± 2.6 μmol/l in controls. Homocysteine level was significantly higher in the patients with lung cancer compared to control group ($p < 0.001$). Mean folate level was 4.3 ± 1.8 pg/ml in cancer cases while 6.1 ± 2.3 pg/ml in controls. That is to say, plasma folate levels were significantly lower in cases of lung cancer compared to controls ($p < 0.001$). There was no significantly difference between groups with regard to B_{12} levels (mean B_{12} level was 234 ± 99 and 240 ± 104 ng/ml in the patients with lung cancer and controls, respectively, $p = 0.78$). Plasma homocysteine, vitamin B_{12} and folate levels did not show significant difference with respect to histologic type of carcinoma. No significant correlation was found between plasma homocysteine, vitamin B_{12}, folate levels and number of cigarettes smoked per day, duration of smoking, age of the patient, and clinical stage of carcinoma. There was also no correlation between number of cigarettes smoked per day, duration of smoking, age of the patient and clinical stage of carcinoma. A possible inverse correlation between plasma homocysteine, vitamin B_{12} and folate levels was not observed. *Conclusion*: In conclusion, high plasma homocysteine and low folate levels could be associated with lung cancer. However, further studies performed on large patient population are needed.**

***Key Words*: homocysteine, vitamin B_{12}, folate, lung cancer.**

Lung cancer is the leading cause of cancer mortality worldwide. In 2014, 224,201 new patients will be diagnosed with lung cancer and 159,260 deaths from lung cancer will occur in the States [1]. A majority of the annual new diagnoses (83.4%) will be dead in 5 years after the diagnosis [2].

Homocysteine (Hcy), a naturally formed non-protein α-amino acid, forms cystathione by combining with serine, which is then hydrolyzed to α-ketobutyrate and cysteine. Hcy is not acquired from the diet, instead it is biosynthesized from and recycled to essential amino acid methionine depending on whether the methionine balance is negative or positive. If the balance is positive, then Hcy is converted to cysteine via transsulfuration through two different enzymes, cystathionine synthase and cystathionase, require vitamin B_6 as a cofactor. On the other hand, in case of shortage of methionine, Hcy accepts a methyl group from N^5-methylthetrahydrofolate (N^5-methyl-THF) in a reaction that uses methylcobalamin, a coenzyme produced from vitamin B_{12}, and Hcy methyltransferase and eventually is converted to methionine. Both cysteine and Hcy concentrations are regulated strictly. Folate and vitamin B_{12} are also involved in DNA methylation as well as in the synthesis of purines and thymidylate for DNA synthesis [3].

Detection of high levels of plasma Hcy, a well-known risk factor for cardiovascular disease, has also been correlated with the development of cancer, in which folate has been suggested to play a pivotal role [4–6]. It was shown that in folate deficient rats DNA breaks and hypomethylation within the p53 tumor suppressor gene occured [7]. Not only does folate deficiency inhibit purine synthesis but it also decreases the cellular concentration of thymine, an essential component of DNA, by preventing methylation of deoxyuridinemonophosphate (dUMP) to deoxythymidinemonophosphate (dTMP) [8]. Additionally, folate regulates DNA methylation by shifting cellular levels of SAM and thus controls gene expression. Global hypomethylation secondary to folate deficiency has been shown in tumor DNA compared with normal tissue DNA [9]. On the other hand, accumulating evidence suggests that hypermethylation results in the suppression of carcinogenesis. DNA methylation changes have

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Abbreviations used: dTMP – deoxythymidinemonophosphate; dUMP – deoxyuridinemonophosphate; Hcy – homocysteine; MN – micronucleus frequency.

been detected in early adenomas of colorectal cancer and prostate cancer [10, 11]. Furthermore, Piyathilake et al. [12] have shown the effects of folate deficiency on DNA hypomethylation in squamous cell lung cancer. In addition to these studies, it has been reported that folate has a protective effect against cervical cancer due to its clear role in DNA synthesis and repair [13].

Because Hcy significantly speeds up oxidation and free radical production, high levels of plasma Hcy have been shown to correlate with chromosomal injury through direct damage of the excess amount of these reactive oxygen products [14]. Mutations in genes, such as p53, occur as a result of DNA oxidation, which in turn might causes cancer development. Additionally, free oxygen radicals might precipitate DNA damage by impairing DNA methylation. Global DNA hypomethylation may lead to loss of chromosomes that results in the formation of micronucleus frequency (MN), which might be used as an indication of genotoxicity and aneuploidy [15].

The correlation between Hcy, relevant vitamins and cancer has been so far investigated mostly in colon, breast and pancreatic cancer [5, 16, 17]. The reports studying this matter on lung cancer are rare. The purpose of this study is to evaluate the levels of Hcy, vitamin B_{12} and folic acid in patients with newly diagnosed lung cancer and determine whether they might be used as an accurate tumor marker for monitoring the patients if they are found to be elevated in lung cancer.

MATERIALS AND METHODS

In this retrospective study, we reviewed the data of 40 men who were diagnosed with lung cancer. All patients were evaluated by imaging techniques (chest radiography or computed tomography). The diagnosis was made via bronchoscopy and biopsy on suspicious lesions. All the patients were newly diagnosed lung cancer patients and have not received any surgical treatment, radiotherapy or chemotherapy. Demographic characteristics of the patients (age, smoking history, occupation, family history of cancer and history of tuberculosis), and histology and stage of the cancer were recorded. Age- and sex-matched 40 healthy controls were enrolled in the study. Hcy, vitamin B_{12} and folate levels were measured in blood samples from the patients and control groups. Patients and the control group avoided medications that had potential effects to alter Hcy levels.

This study was approved by local ethical committee. Informed consent was obtained from each subject in the hospital.

Blood sampling. 3 ml of blood were drawn from antecubital veins of both the patients and the control group in the morning before breakfast and they were put into K3 EDTA tubes. K3 EDTA tubes were then centrifuged at 2500/g for 5 min. No hemolysis occurred in the samples. Plasma samples were removed and transferred to an eppendorf tube. Examples were numbered and stored at −80 °C until analysis.

Analysis of plasma Hcy. Plasma Hcy levels were measured by High Performance Liquid Chromatography (HPLC) method. 100 µl of plasma, 25 µl of internal standard and 25 µl of reduction reagent were mixed in a 1.5 ml plastic eppendorf tubes. Stirring gently, the mixture was incubated at room temperature for 5 min. Then 100 µl precipitant reagent was added and mixed for 30 s. The mixture was centrifuged at 9000/g for 7 min. 50 µl supernatants of all samples were taken to another eppendorf tube and 100 µl of derivatizing reagent were added. It was incubated at 55 °C for 10 min. It was removed from the water bath and transferred to the sample vessels. HPLC equipment was programmed to take 20 µl of the supernatant automatically. Analysis of each sample was completed in 6 min. Results were given as µmol/l.

The Intra-assay and Inter-assay Reproducibility Study. Each parameter was studied for 10 times consecutively in relation to the intraday reproducibility study for Hcy. For the inter-day reproducibility, each parameter was studied for 10 times in different days or different sessions on the same day.

Other biochemical analysis. Vitamin B_{12} and folic acid levels of patient and control group were measured by Immulate DPC Immunoassay (Los Angeles, USA) device with using Immulate DPC kits.

Statistical analysis. Statistical evaluations of the findings obtained from our study were performed in SPSS 18.0 software package. The mean values and standard deviation of the parameters were calculated. Student *t*-test was used to assess the differences between the two groups while the differences between the three groups were evaluated with ANOVA test. Pearson's correlation test was performed for the correlation analysis. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The study included 40 male patients with lung cancer and age-matched 40 healthy men. The mean age of the patients was 58.7 ± 9.9 years (median: 58, range: 38–78). All of the cases were smokers. The average of the daily cigarette consumption was 2 ± 0.7 packs (median: 2, range: 1–4), and the average numbers of years of smoking was 30 ± 11 (median: 29, range: 10–58).

Of the patients, 55% had squamous cell carcinoma, 35% had adenocarcinoma and 10% had small cell carcinoma. 20% of the cases were in stage II, 20% were in stage IB, 2.5% were in stage III, 10% were in stage IIB, 12.5% were in stage IIIA, 20% were in stage IIIB and 15% were in stage IV.

The mean Hcy level of the patients with lung cancer was 15.3 ± 7.3 µmol/l (median: 14.8 and range: 5.0–36.2), while it was 9.8 ± 2.6 µmol/l (median: 10.1 and range 4.2–13.4) in the control group. Hcy levels were significantly higher in lung cancer patients compared with the control group ($p < 0.001$). The mean plasma folate levels were 4.3 ± 1.8 pg/ml (median: 3.8 and range: 1.9–7.9) in patients with lung cancer

whereas it was 6.1 ± 2.3 pg/ml (median: 6.1 and range: 2.4–11.5) in the control group. Accordingly, the plasma folate levels were significantly lower in lung cancer patients than in controls ($p < 0.001$). No significant differences were detected between the two groups in terms of vitamin B_{12} levels (for patients with lung cancer mean: 234 ± 99 ng/ml, median: 226, range: 154–797; for the controls mean: 240 ± 104 ng/ml, median: 208, range: 100–539 and $p = 0.78$). The levels of plasma Hcy, folate and vitamin B_{12} in cancer patients and controls were shown in Table. No difference was observed in the plasma Hcy, folate and vitamin B_{12} levels according to histological type.

Table. Plasma Hcy, folate and vitamin B_{12} levels in lung cancer patients and the healthy controls

Indexes	Patient group		p
	lung cancer patients (n = 40)	controls (n = 40)	
Hcy, μmol/l	15.3 ± 7.3	9.8 ± 2.6	< 0.001
Folate, pg/ml	4.3 ± 1.8	6.1 ± 2.3	< 0.001
Vitamin B_{12}, ng/ml	234 ± 99	240 ± 104	0.78

Note: values were shown as mean ± SD.

There was not a significant correlation between plasma Hcy, folate and vitamin B_{12} levels and number of cigarettes smoked daily, the total duration of smoking, age and stage of cancer. There was not a significant relationship between the disease stage and the number of cigarettes smoked daily, the total duration of smoking and age of the patients. Likewise, a significant inverse correlation was not detected between plasma Hcy levels and folate, vitamin B_{12} levels in contrast to expected. As predictable, a significant positive correlation was present between age and duration of smoking ($p = 0.001$).

There have been numerous studies investigating the association between cancer and serum vitamins B_6, B_{12}, folate and Hcy. Deficiency in folate has been shown to increase the risk of cervical, colorectal, lung, esophagus, brain, pancreas and breast cancer in epidemiological, clinical and experimental studies [18]. Large prospective epidemiological studies have determined an inverse dose-dependent relationship between dietary folate intake and cancer risk; that folate supplementation could reverse cervical dysplasia and moderately increased amount of dietary folate or sufficient serum folate levels reduced the risk of pancreatic and breast cancer [16, 17, 19, 20]. However, in a recently published metaanalysis of data on 50,000 individuals concluded that folic acid supplementation had no impact on overall and site-specific cancer incidence [21].

Studies suggest that there is an inverse correlation between folate status in the body and the development of lung cancer [22]. Two randomized controlled trials have shown improvement of atypia in patients with bronchial squamous cell metaplasia with folate and vitamin B_{12} therapy. In addition, plasma folate levels in smokers with metaplasia were significantly lower than those in smokers without metaplasia [23, 24]. Bandera et al. [25] (New York State Cohort Study) have investigated the relationship between the risk of lung cancer and diet. This cohort study in which 27,544 men and 20,456 women participated, men with low amount of dietary vitamin C, carotenoids and folate intake were found to have increased risk of lung cancer. Similar results have been identified by Voorrips et al. [26] in a large epidemiological study (Netherlands Cohort Study). Furthermore, in a metaanalysis it has been determined that both folate intake and high serum folate levels were significantly associated with reduced risk of lung cancer [27]. On the other hand, in a recently published metaanalysis of 9 cohort studies including 566,921 individuals have shown that folate intake did not have as much impact on the risk of lung cancer as expected. Another suprising result of this study was that low folate intake might have a protective role on women whereas high folate intake plays a protective role on men [28]. However, a 16-year prospective cohort study on 1,793,327 women (Nurses' Health Study) has failed to show an association between lung cancer and the amount of folate in the diet [29]. Similarly, the metaanalysis of the randomized controlled trials have pointed out that folic acid supplementation has no significant effect on overall cancer incidence [30].

Pyridoxal-5´-phosphate (PLP), the bioactive form of vitamin B_6, is substantial for the catabolism of Hcy. Thus, reduced circulating levels of B_6 vitamin result in hyperhomocysteinemia. It has been identified that higher levels of pyridoxal kinase (PDXK), the enzyme that activates B_6 vitamin by converting it into PLP, is a therapy-independent prognostic marker in patients with non-small cell lung carcinoma [31]. The double-blind, placebo-controlled study by Hartman et al. [32] has found that the risk of lung cancer was significantly lower in men with higher vitamin B_6 levels. It was the first report of vitamin B_6 role in lung cancer. In addition, in a population-based, prospective cohort study including 74,941 women who have never smoked, it was reported that dietary riboflavin intake and a higher than median intake of methionine were inversely associated with lung cancer risk whereas no such relationship was found with other B vitamins [33]. Similar results were shown for riboflavin in current smokers in the Melbourne Collaborative Cohort Study that comprised 41,514 indiduals whereas there was no such correlation in individuals who previously smoked and who never smoked, and there was no such correlation for other B vitamins or for methionine [34]. On the other hand, in the European Prospective Investigation into Cancer and Nutrition (EPIC) study including 519,978 participants, higher serum levels of B_6 and methionine were associated with reduced lung cancer risk in all of never, former, and current smokers while lower risk of lung cancer was correlated with higher serum folate only in former and current smokers [35]. In contrast to these findings, in Norwegian Vitamin Trial and Western Norway B Vitamin Intervention Trial including 6,837 patients with ischemic heart disease, folic acid plus vitamin B_{12} intake was correlated with increased cancer incidence, cancer mortality (especially in lung cancer), and all-cause mortality [36].

The present study showed that plasma folate levels were significantly lower in patients with newly diagnosed lung cancer compared to age- and sex-matched healthy controls. This result supports the suggestion that reduced folate status is associated with increased risk of lung cancer. However, no such difference was detected for vitamin B_{12} levels.

In a current study, a structural equation modeling put together four different kinds of variable including methionine-homocysteine metabolism, folate cycle, transsulfuration, and immune activation and pointed out their complex relationship with each other in lung cancer carcinogenesis [37]. In that study, it was argued that methionine-homocysteine metabolism and immune activation had a stronger association with the lung cancer risk than did the other potential mechanisms. In another study, it was shown that increased cancer-related death risk was significantly associated with high Hcy levels [4]. Furthermore, cervical intraepithelial neoplasia was found to be in a positive correlation with high Hcy levels [6]. The patients with Hcy levels in the highest quartile (12.2 mmol/l) have been reported to have increased cancer risk by 70% compared to patients with the lowest quartile (⩽7.9 mmol/l) [5]. It was shown that disorders in Hcy metabolism were associated with colon, breast and pancreatic cancers [5, 16, 17, 19].

In our study, the finding that plasma Hcy levels in lung cancer patients were significantly higher than those in controls, has supported the view that hyperhomocysteinemia was an important risk factor for lung cancer development. Although serum Hcy concentration was shown to be inversely proportional to serum folate, vitamins B_6 and B_{12} levels in a previous study, there was not a significant inverse relationship between Hcy and folate or vitamin B_{12} levels in our study [38]. In another study, in accordance with our results a significantly higher t-Hcy, lower t-Glutathion and folate levels were found in the advanced-stage group compared with those in the control group [39]. In our study, the result that the levels of folate were low while vitamin B_{12} levels were normal might be explained by the fact that folate was a more important factor in Hcy metabolism than vitamin B_{12}.

High Hcy level in patients with lung cancer might be used as a sensitive marker for early diagnosis and detection of recurrences. However, since Hcy rises in other tumors types and the sensitivity and specificity of Hcy have not been determined in this study, a more precise statement is yet to be made.

A correlation between plasma Hcy, folate, vitamin B_{12} levels and the number of cigarettes smoked per day, total time of smoking and the histological type of cancer could not be detected. Bandera et al. [25] found in a fairly large population that lower folate levels increased the risk of lung cancer. This increased risk was more associated with squamous cell cancer compared to adenocarcinoma. Furthermore, this risk was increased more significantly in people who consumed a larger amount of cigarettes (>20/day). In our study, in contrast to these results, no relationship between folate and either smoking or histological type of the lung cancer was determined.

In conclusion, significantly higher plasma Hcy and lower folate levels was found in lung cancer patients compared to controls whereas there was no difference in B_{12} levels between two groups. In order to evaluate the value of plasma Hcy levels in lung cancer as a marker, further studies with larger number of patients are required.

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