Usefulness of Homocysteine as a Cancer Marker: Total Thiol Compounds and Folate Levels in Untreated Lung Cancer Patients

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Abstract. *Background: Apart from being a risk factor for atherosclerotic cardiovascular diseases, the latest research suggests homocysteine as a marker for cancer. We aimed to explore the clinical utility of plasma homocysteine levels as a marker in lung cancer. Patients and Methods: Changes in serum total thiols and folate levels were investigated in newly diagnosed untreated lung cancer patients (n=37) and compared with healthy controls (n=26). Fluorometric HPLC methods were used for the determination of thiols. Other parameters were determined with commercial diagnostic kits. Results: Increased total homocysteine (t-Hcy), decreased total glutathione (t-GSH) and folate levels were observed in lung cancer patients compared with healthy controls. Total levels of thiols and folate did not show any significant difference between SCLC and NSCLC patients. However, there were significantly higher t-Hcy, lower t-GSH and folate levels in the advanced-stage group compared with controls. Prevalence of hyperhomocysteinemia was 65% in lung cancer patients when 12 μmol/l were taken as a cut-off value for t-Hcy levels. Conclusion: Homocysteine is suggested as a marker for several types of cancer, but our result did not support this hypothesis for lung cancer. Although higher homocysteine levels were observed in the present study, further investigation in the larger cancer population would clarify the importance of homocysteine as a cancer marker.*

Homocysteine has attracted great interest for researchers in the last decade. In spite of evidence for a role of homocysteine in pathogenesis of cardiovascular diseases (1), renal failure (2), pregnancy complication (3), psychiatric and neurodegenerative disorders (4) the mechanisms by which homocysteine can be harmful to the cells are largely unclear.

Homocysteine is a branch-point intermediate resulting from the methionine metabolic cycle and undergoes transsulfuration or transmethylation reactions depending on metabolic demand. The transsulfuration pathway results in the formation of cysteine, a precursor of glutathione. The transmethylation reaction requires 5-methyl-tetrahydrafolate as a methyl donor and methionine is resynthesized from S-adenosyl-homocysteine (SAH) in this pathway (5). Due to the critical role of folate in all transmethylation reactions, genetic defects of the enzymes and deficiencies of cofactors included in these pathways and the folate cycle influence plasma total homocysteine levels (5, 6).

Malignant cells are characterized by high a growth rate, and the methionine requirement increases in these cells due to increased protein synthesis and transmethylation reactions. Normal cells meet their methionine requirement by synthesizing it from homocysteine. In contrast, methionine-dependent malignant cells in organs such as the lung, kidney, breast, colon and bladder cannot convert homocysteine to methionine, which results in homocysteine accumulation (7). An increase in the level of homocysteine is also associated with folate deficiency because folate cofactors act as essential intermediates in homocysteine remethylation, S-adenosylmethionine (SAM) synthesis (which is the most important methyl donor) and in the production of purine and thymidine for DNA synthesis (8).

Early detection of lung cancer is highly difficult, therefore advanced and metastatic stages at diagnosis are characteristics of lung cancer patients. For this reason, it is of great importance to find biomarkers that can detect lung cancer at early stages and that make evaluation of responsiveness of therapy at advanced stages easy. Studies showed that patients with ovarian (9), pancreatic (10), colorectal (11), head and neck squamous cell carcinomas (12, 13) and acute lymphoblastic leukemia (14) had higher

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Table I. *Baseline characteristics and some biochemical parameters in lung cancer patients and healthy controls.*

	Patients (n=37)	Controls (n=26)	*p*
Age (years)			
Mean	60.8	60.7	ns
Range	45-78	40-77	
Gender (F/M)	5/32	5/21	
Body Mass Index (kg/m^2)[a]	24.5±4.7	25.3±3.9	ns
Alcohol intake, n (%)			
Yes	3 (8)	5 (19)	
No	34 (92)	21 (81)	
Smoking frequency, n (%)			
Never smoked	4 (11)	5 (19)	
Ex-smoker	8 (22)	13 (50)	
Current smoker	25 (67)	8 (31)	
Sedimentation rate (mm/h)[a]	63.0±27.4	21.6±18.5	0.0001
C-reactive protein (mg/l)[a]	69.4±42.8	9.7±10.3	0.0001
Glucose (mmol/l)[a]	5.7±1.5	5.8±0.6	ns
Total cholesterol (mmol/l)[a]	5.2±1.2	5.3±0.9	ns
Triglyceride (mmol/l)[a]	1.2±0.5	1.4±0.5	ns
Total protein (g/l)[a]	72.1±6.8	75.9±4.4	0.012
Albumin (g/l)[a]	36.6±3.8	37.9±1.5	ns
Creatinine (μmol/l)[a]	61.9±17.7	70.7±8.8	ns

[a]Values shown are the mean±standard deviation.

homocysteine levels. In addition, there was an increase in homocysteine levels in rapid proliferating tumor cell lines and plasma homocysteine levels declined when cells started dying (10). As a result of these studies, it was suggested that the plasma homocysteine level may be used as a potential tumor marker.

In our study, we aimed to determine total plasma homocysteine levels in newly diagnosed, untreated patients with different types and stages of lung cancer, to evaluate their relation to other thiol compounds and folate levels, and also to explore the clinical utility of plasma homocysteine levels as a cancer marker.

Patients and Methods

Study population. Thirty seven patients suffering from lung cancer, enrolled at the Department of Chest Diseases of the Social Insurance Hospital of Ankara, and twenty six age and sex-matched healthy control subject were included in this study. All patients had been newly diagnosed by bronchoscopic punch biopsy and (or) bronchial lavage cytology and had had no any clinical or radiological anticancer treatment at the time of the study. The control group was selected from subjects having no known disease effecting homocysteine levels, nor taking any medication or vitamin supplementation. Both the patients and controls had normal renal function. Baseline characteristics and some biochemical parameters in patients and controls are given in Table I. The classification of patients was done according to the TNM classification (15). Of 37 patients, 9 had small cell lung cancer (SCLC) and 28 had non-small cell lung cancer (NSCLC). Patients were in stage IB, IIB (n=2), stage IV (n=7) for SCLC; stage IB (n=6), stage IIB (n=1), stage IIIA (n=2), stage IIIB (n=2), stage IV (n=17) for NSCLC. The study protocol was approved by the Local Ethical Committee of the Hospital in accordance with the ethical standards of the Helsinki Declaration.

Table II. *Mean serum values of total homocysteine, cysteine, glutathione and folate in healthy controls and patients with SCLC and with NSCLC.*

	Homocysteine (μmol/l)	Cysteine (μmol/l)	Glutathione (μmol/l)	Folate (nmol/l)
Controls	11.7±2.5	310.4±63.9	3.6±1.8	28.7±10.4
SCLC (n=9)	16.0±7.3	297.3±59.6	2.9±1.8	22.2±13.4*
NSCLC (n=28)	13.8±4.3	307.3±58.2	2.0±1.4**	15.9±5.9***

*$p<0.05$, **$p<0.001$, ***$p<0.0001$ compared with controls.

Methods. Total homocysteine, cysteine and glutathione were measured using fluorometric HPLC methods with some modification (16, 17). These methods depend on the derivatization of thiols with 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) after reduction with tris-(2-carboxyethyl)phosphine (TCEP). Briefly, 200 μl of serum sample were mixed with 20 μl of TCEP (120 g/l in phosphate buffer saline) and left at 37˚C for 10 min to reduce disulfide bounds. The samples were then mixed with 125 μl of 0.6 mol/l perchloric acid containing 1 mmol/l EDTA and left at room temperature for 5 min. Precipitated proteins were removed by centrifugation at 15,000 xg for 10 min. Clear supernatant (100 μl) was mixed with derivatization solution containing 200 μl of potassium borate buffer (2 mol/l, pH 10.5) and 100 μl of 7-fluoro-benzo-2-oxa-1,3-diazole-4-sulphonate solution 1 g/l in the potassium borate buffer (0.1 mol/l, pH 9.5). The samples were incubated at 60˚C for 30 min in dark. After derivatization, samples were cooled on ice and applied to an HPLC system (HP 1050 series, Avondale, PA, USA). Supelco™ LC_{18} DB (15 cm x 4.0 mm I.D., 3 μm) analytical column and Supelguard™ LC_{18} DB (2 cm) guard column were used in the analysis. Acetate buffer (0.1 mol/l, pH 4) containing 2% methanol was used as a mobile phase. The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm.

Serum folate levels were measured by radioimmunoassay (DPC, Los Angeles,USA), according to the manufacturer's instructions. Other biochemical parameters were measured by using commercial diagnostic kits. Total protein and albumin levels were determined colorimetrically with commercial kits (Stanbio, USA). The erythrocyte sedimentation rate was determined by Westergreen test. C-reactive protein levels were measured nephelometrically (Dade Behring, Liederbach, Germany). Creatinine levels were determined on a Hitachi 912 analyzer (Roche Diagnostics, GmbH, Manheim, Germany). Total cholesterol and triglycerides were determined enzymatically (Sigma, St. Louis, USA).

Statistical analysis. Statistical analysis was performed using the SPSS 8.0 software (SPSS, USA). Data were presented as mean±S.D. The Mann-Whitney *U*-test, Kruskal-Wallis variance analysis and Spearman's rank correlation analysis were applied. A *p*-value <0.05 was considered statistically significant.

Table III. *Serum homocysteine, cysteine, glutathione and folate levels in lung cancer patients, grouped according to stages, and in the controls.*

		Lung cancer patients			
	Controls	Stage I	Stage II	Stage III	Stage IV
Homocysteine μmol/l	11.7±2.5	11.9±2.0****	23.1±15.3***	14.6±4.3	14.1±4.4**
Cysteine μmol/l	310.4±63.9	345.2±65.9	246.5±50.5	306.3±56.5	297.8±52.3
Glutathione μmol/l	3.6±1.8	1.8±0.7	4.4±3.7	2.4±1.1	2.0±1.4**
Folate nmol/l	28.7±10.4	21.3±6.8	32.4±27.6	13.4±7.4	16.1±5.4*

*$p<0.02$ compared with controls, **$p<0.03$ compared with controls, ***$p<0.002$ compared with controls, ****$p<0.007$ compared with Stage II.

Results

The mean total serum homocysteine value was higher in patients (14.2±5.2 μmol/l) than in controls (11.7±2.5 μmol/l), but did not reach statistically significant levels ($p=0.056$). Total cysteine levels did not show any statistically significant difference between groups, but total glutathione and folate levels of patients (2.2±1.5 μmol/l and 17.4±8.6 nmol/l, respectively) were significantly lower than those of controls (3.6±1.8 μmol/l and 28.7±10.4 nmol/l, respectively) ($p<0.001$ and $p<0.0001$, respectively). When 12 μmol/l t-Hcy was used as a cut-off value, hyperhomocysteinemia prevalence in patients was 65%. Total serum homocysteine, cysteine and glutathione levels of patient groups (SCLC and NSCLC groups) and control subjects are detailed in Table II. There was no significant difference between SCLC patients and NSCLC patients in terms of t-Hcy, t-Cys, t-GSH or folate levels.

As shown in Table III, when we evaluated the parameters according to disease stage, total cysteine, glutathione and folate levels did not show any significant differences among the stage groups. However, total homocysteine levels of patients in stage II were higher than those of controls ($p<0.002$). Patients in stage I and stage IV had significantly lower homocysteine levels compared with patients in stage II ($p<0.007$ and $p<0.03$, respectively). Serum folate levels were significantly lower only in patients in stage IV compared with controls ($p<0.02$).

When patients were divided into two groups -an early stage group including stage I and II and advanced stage group including stage III and IV-, higher total homocysteine ($p<0.04$), lower total glutathione ($p<0.003$) and folate ($p<0.001$) levels were observed in the advanced stage group compared with the controls. There was also a significant decrease in total glutathione levels in the early stage group compared with the controls ($p<0.02$) (Figure 1).

Homocysteine levels were negatively correlated with folate levels in both control and NSCLC groups (r=–0.513, $p<0.008$; r=–0.485, $p<0.02$, respectively). There was also negative correlation between total cysteine and folate levels in controls (r=–0.504, $p<0.01$). There was a positive correlation between total cysteine and homocysteine in controls (r=0.673, $p<0.0001$), but this correlation disappeared in patients.

Discussion

DNA methylation plays an important role in tissue- and development-specific gene regulation which is essential for the control of normal cell growth, differentiation, and apoptosis. Microarray-based studies showed that methylation disturbances are frequent in lung cancer and that global DNA hypomethylation and regional hypermethylation contribute to cancer initiation and progression (18).

Methionine dependence is a phenotype characteristic of tumor cells. It was shown that methionine-dependent cells have a low SAM/SAH ratio compared with methionine-independent cells (7). Methionine cycle disruptions, by reducing intracellular SAM levels, can alter cytosine methylation in DNA leading to activation of protooncogenes, repression of tumor suppressor genes and induction of malignant transformation (19). SAH, the product of methylation reactions, subsequently hydrolyses to homocysteine. Increased SAH levels lead to an increase in homocysteine levels as long as homocysteine is not converted to cysteine by transsulfuration pathways. We observed higher homocysteine and unchanged cysteine levels in patients with lung cancer, especially in the advanced stage group. This is in accordance with previous studies (9, 11, 12, 14) implying that hyperhomocysteinemia could be phenotypic expression of malignancy. Sun *et al.* (10) showed that homocysteine levels increased in different tumor cell lines (A549, human lung cancer; SK-N-AS, human metastatic neuroblastoma; MCF-7, human breast adenocarcinoma) compared with normal cell lines. This increase is linked to folate depletion and rapid tumor proliferation. In our study, we observed lower folate levels and higher t-Hcy levels in the advanced stage group, in accordance with their observation. It was also shown that genetic variation in folate metabolism and reduced folate intake was associated with an increased risk of lung cancer (20, 21). In previous studies, maintenance of

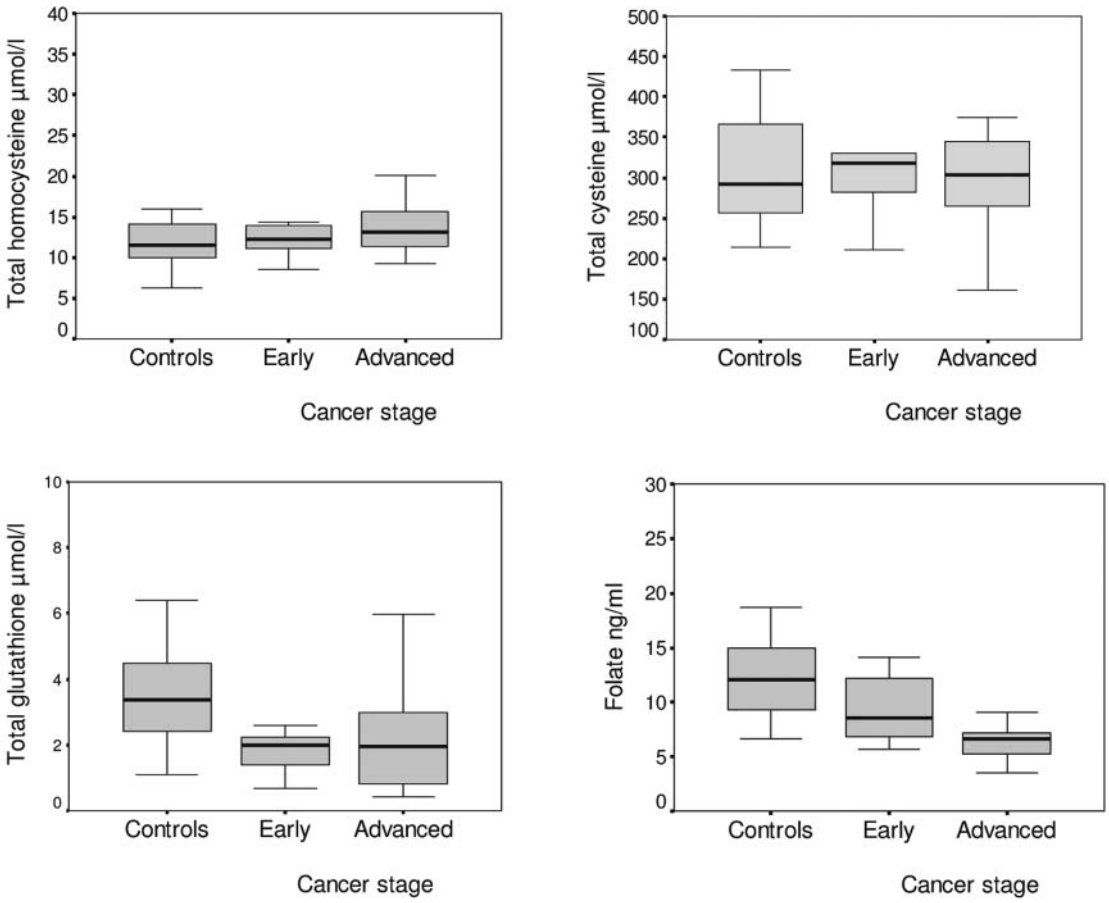

Figure 1. *Serum of total homocysteine, cysteine, glutathione and folate in patients with early stage (stage I+II), advanced stage (stage III+IV) and controls.*

adequate folate status has been associated with a protective effect and reduced incidence of several types of cancer including lung cancer (21-26). In our study, we observed statistically significant decreases in folate levels in both SCLC and NSCLC patients, with this decrease being more significant in the advanced stage group. Moreover, folate levels significantly correlated with homocysteine levels. These results imply that increased homocysteine levels are related to folate depletion or deficiency, as well as disturbed methylation pathways. Homocysteine itself is toxic to cells. Oxidation of thiol-containing amino acids produces free radicals having damaging effects on DNA (27), and their pro-oxidant effects have been implicated in DNA damage (28). Decreased glutathione levels in our study indicate increased detoxification against oxidative stress in carcinogenesis. This observation is also in accordance with previous studies (29, 30). Homocysteine becomes toxic to human cells via its highly reactive lactone form which is incorporated into protein by methionyl-tRNA synthase (31). Therefore, determination of homocysteine levels is also important for protection from its toxic effects and for choosing therapy.

Although we observed a higher prevalence of hyperhomocysteinemia in lung cancer patients in our study, this is not sufficient to accept homocysteine as a cancer marker; we think that determination of homocysteine in a larger cancer population is important to clarify the usefulness of homocysteine as a marker of cancer and effectiveness of drug therapy. Further studies in larger population are needed to clarify these points and to determine an appropriate cut-off value of homocysteine for cancer patients.

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