Plasma homocysteine levels in patients with esophageal cancer

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Özet

Özofagus kanserli hastalarda plazma homosistein düzeyleri

Amaç: Kanser tüm dünyada en önemli sağlık problemlerinden birini oluşturmaktadır. Ölüme neden olan hastalıklar arasında kalp hastalıklarından sonra ikinci sırayı almaktadır. Tümör belirteçleri kanserlerin tanısında ve tedaviye cevabın değerlendirilmesinde kullanılmasına rağmen henüz herhangi bir kanser türü için özgül bir belirteç bulunamamıştır. Hiperhomosisteinemi bir çok hastalıkla ilişkili bulunmuştur. Yapılan değişik çalışmalarda bazı kanser türlerinde de artış tespit edilmiştir. Bu çalışmada özofagus kanserli hastalarda homosistein düzeyleri ve diğer tümör belirteçleri arasındaki ilişki araştırıldı. Methods: Bu çalışmaya 2003 0cak ve 2004 Temmuz tarihleri arasında hastanemizde endoskopik biopsilerle histopatolojik tanısı konmuş toplam 40 özofagus kanserli hasta grubu ile 40 sağlıklı kişiden oluşan kontrol grubu alındı. Tümör belirteçlerinden Homosistein, CEA ve CA19-9 düzeyleri özefagus kanserli hastalar ile sağlıklı kontrol grubu olmak üzere iki grup üzerinde çalışıldı. Veriler istatistiksel olarak karşılaştırılarak özofagus kanserli olgularda homosistein seviyesinin önemi araştırıldı. Bulgular: Kontrol grubu ile kıyaslandığında özofagus kanserli hastalarda plazma homosistein (hcy) düzeyleri anlamlı derecede yüksek bulundu ($p<0.05$). Yine aynı vakalarda çalışılan tümör belirteçlerinde gözlenen artış kontrol grubu ile kıyaslandığında istatistiksel açıdan anlamlı değildi. Bununla birlikte özofagus kanserli hasta grubunda hcy düzeyleri ile tümör belirteçlerinin düzeyleri arasında herhangi bir korelasyon yoktu. Sonuçlar: Plazma homosistein düzeyleri özofagus kanserlerinde artmaktadır ve tümör belirteci olarak kullanılabileceğini düşünüyoruz. Ancak daha geniş kapsamlı çalışmalara ihtiyaç vardır.

Anahtar kelimeler: Homosistein (hcy), GİS maligniteleri, tümör belirteçleri.

Abstract

Objectives: Cancer accounts for one of the most important health problems in the whole world. Among fatal diseases, it is ranked second following cardiovascular diseases. Though tumor markers are used in the diagnosis of cancers and response to treatment, yet no marker specific to a type of cancer has been found. Hyperhomocysteinemia has been found to be related to several diseases. In various studies conducted, increments in plasma homocysteine have been observed in some cancer types. In this study, the relationship between homocysteine levels and other tumor markers in esophagus cancer patients was investigated. Methods: A total of 40 patients with esophagus cancer, diagnosed histopathologically with endoscopic biopsies in our clinic, and 40 healthy subjects enrolled in this study. The levels of tumor markers homocysteine, CEA and CA19-9 were investigated in the two groups, namely the esophagus cancer patients and healthy controls. The significance of homocysteine levels in esophagus cancer patients was studied via statistical comparison of the data. Results: As compared with the controls, the plasma homocysteine levels were significantly higher in esophagus cancer patients ($p<0.05$). The increments observed in other tumor markers studied in the same cases were not statistically significant when compared with the controls. However, no correlation was found between the homocysteine and other tumor marker levels in the esophagus cancer patients. Conclusion: Plasma homocysteine levels increase in esophageal cancers and thus it may possibly be used as a tumor marker. However, further large-scale studies are required for this hypothesis.

Key words: Homocysteine, esophageal carcinoma, tumor markers

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Introduction

Tumor markers are enzymes, metabolites, immunoglobulins, various proteins and tumor-associated antigens that are synthesized by neoplastic and embryonic cells, and used in the medical fields of screening, classification, localization, diagnosis and follow-up of cancers, evaluation of response to treatment and determination of relapses. However, these substances are also synthesized in normal tissues, and may be found in the circulation, body fluids, cell membranes and cytoplasm. The first tumor marker discovered was the Bence Jones protein described in the urine in 1846 and used in multiple myeloma patients. However, tumor markers first became useable in clinics following the discoveries of AFP by Abelev in 1963 and CEA by Fredman in 1965 (1-3). The diagnostic value of tumor markers depends on their sensitivity (i.e. epidemiological ability of the tumor marker to detect cancer) and specificity (i.e. ability to distinguish the non-cancerous population). The ideal sensitivity and specificity values are 100%. Despite numerous extensive studies conducted, no tumor marker with full sensitivity for any cancer has yet been found.

Homocysteine (Hcy) is an amino acid with a free thiol (sulphydryl) group. Hcy is not present in the diet. The only source of Hcy in the body is methionine, an essential amino acid. Hcy does not contribute to the structure of proteins but is valuable in being the source of important intermediate products (4,5). De Vigneaud first discovered the presence of intermediate products in amino acid metabolism in 1932 (6,7). In recent epidemiological studies, a relationship between Hcy levels and coronary, cerebral and peripheral atherosclerosis, venous thromboembolism, and neural tube defects has been established. A fasting plasma tHcy concentration higher than 15 mol/L is referred to as hyperhomocysteinemia, and this is classified according to Hcy concentrations of 15-30 mol/L, 30-100 mol/L and 100-500 mol/L as mild, moderate and severe hyperhomocysteinemia respectively. This study aims to compare tumor markers and plasma Hcy levels in patients with GIS malignancy and determine whether plasma Hcy levels may or not be used as tumor markers.

Methods

Forty patients with esophageal cancer, admitted to the Internal Medicine and Thoracic Surgery clinics in the Faculty of Medicine in Ataturk University, diagnosed histopathologically with endoscopic biopsies in the respective clinics were included in this study. As for the controls, 40 subjects who had no health complaints, physical examination signs, risk of malignancy, infections or systemic diseases determined clinically or in laboratory tests, and who did not receive drugs that may influence the Hcy levels, were included. B_{12} and folic acid levels were assessed in order to exclude cases of vitamin deficiency. Besides, each case was investigated with imaging techniques including ultrasonography and computed tomography. All of these patients were newly diagnosed, and had not undergone surgery, chemotherapy or radiotherapy. As tumor marker tests, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels were assessed in the sera of patients and controls.

Two groups, one composed of esophageal cancer cases and the other of controls were formed.

Group I: This group included 40 patients with esophageal cancer, aged 28-82. Of these patients, 23 were males and 17 were females, and histopathologically 34 patients had squamous cell carcinoma and 6 had adenocarcinoma.

Group II: The control group consisted of 40 healthy subjects aged 22-80. Of these patients, 25 were males and 15 were females.

Specimen Collection

From every participant in the study, 3 ml of early morning fasting blood obtained from the antecubital region was taken into a test tube containing K3 EDTA, which was centrifuged within half an hour at 2500g for 5 minutes. Specific attention was paid to absence of hemolysis in the specimens. Plasmas of the specimens were extracted into Eppendorf tubes, and stored at -80C until analyses.

Plasma Homocysteine Analysis

The plasma Hcy levels were assessed via the HPLC method (8). In order to perform this, 100 L of plasma, 25 L of internal standard and 25 L of reduction reactant were placed in 1.5 ml plastic Eppendorf tubes. This mixture was incubated with mild mixing at room temperature for 5 minutes. Then, 100 L of precipitation reactant was added and mixed for 30 seconds. The final mixture was centrifuged for 7 minutes at 9000g. From the supernatant of each specimen, 50 L was taken into a different Eppendorf tube and 100 L derivatization reactant was added. The final mixture was suspended for 10 minutes at 55C, and removed from the water bath in order to be

transferred to the specimen containers. The HPLC device was programmed to take 20 L of the supernatant automatically. Analysis of each specimen was finished in 6 minutes. The results were expressed as mol/L.

Intra-assay and Inter-assay Reproductability Studies

Each parameter regarding same-day reproductability studies for homocysteine was assessed ten consequent times. For reproductability between days, each parameter was assessed ten times on different days or at different sessions on the same day.

Tumor Marker Studies

5 ml of early morning fasting blood was drawn from the forearm with a disposable injector from each member of the patient and control groups. The samples were transferred to standard biochemistry tubes, and left to clot at room temperature for 20 minutes. Following clotting, each blood sample was centrifuged for 5 minutes at 5000 rpm. The removed serum was transferred into a dry tube. The CEA levels were assessed using the chemiluminometric method (IMMULITE-2000 DPC-Diagnostic-USA). Likewise, the CA 19-9 and AFP levels were assessed using the same method (HITACHI E 170 Tokyo-Japan). The normal values of the tumor markers mentioned were as follows: CEA: 0-4 ng/ml, CA 19-9: 0-33 u/ml, AFP: 0-5.8 IU/ml.

Other Biochemical Analyses

The levels of vitamins B_{12} and folic acid in the patients and controls were assessed using Immulate DPC kits and an Immulate DPC Immunoassay device (Los Angeles, USA). Patients whose folic acid and vitamin B_{12} levels were not normal were excluded from the study.

Statistical Analyses

The results obtained at the end of our study were evaluated statistically. The results were expressed as means standard deviations (X SD) or as means standard errors (X SE). The statistical analyses were conducted using SPSS for Windows 11.0 software in computerized environment and p values less than 0.05 were predetermined as statistically significant. The differences between group means were determined with analysis of variance (ANOVA) for degrees of significance. Pearson correlation test was used for analysis of correlation.

Results

As compared with the controls, the Hcy levels in esophageal cancer patients were found to be significantly higher ($p<0.05$). However, the increments in tumor markers studied in the same cases were not statistically significant when compared with the controls. Besides, there was no correlation between the Hcy and tumor marker levels in the esophageal cancer patients. The cumulative data of the patients and controls are displayed in Table 1.

Table 1. The mean (X) and standard error (SE) values of the parameters studied in the patient and control groups

	Esophageal cancer group	Control group	p
Cases (n)	40	40	NS
Age	61,7±13,2	60,6±7,7	NS
Hcy	14,3±2,1*	8,5±0,4	<0.05
CEA	4,1±1,1	2,6±0,3	NS
CA 19-9	30,5±7,1	16,7±2,8	NS
AFP	2,1±0,3	1,8±0,2	NS

*$p<0.05$ versus the control group

Discussion

Cancer continues to be one of the most important health problems in the world, placed second among all fatal diseases after cardiovascular diseases. About 20% of cancer-related deaths in the world are related to GIS malignancies (9). Therefore, early diagnosis of cancers significantly influences both morbidity and mortality.

Tumor markers are used in medical fields in the screening, diagnosis, localization, classification, staging and follow-up of cancers and in evaluation of response to treatment and determination of relapses. However, these substances are also synthesized in normal tissues. Sir Henry Bence Jones was the first to describe a protein, known today with his name, in multiple myeloma patients in 1846. Although multiple tumor markers have been used since that time, yet no tumor marker has been found to be completely sensitive to a type of cancer (1-3).

For GIS tumors, the CEA and CA 19-9 tumor markers have become indisputable in clinical practice. In a study conducted in Germany, it has been shown that 98.5% of internists and 92.6% of surgeons employed CEA and CA 19-9 particularly in the diagnosis and follow-up of GIS malignancies (10).

Homocysteine (Hcy) is an amino acid with a free thiol group. It is particularly valuable in that it is the source of important intermediate products. Hyperhomocysteinemia is among important risk factors in cardiovascular disorders. Besides, it has

been reported in several diseases, including malignancies (11-13). Hcy values are thought to cause genetic instability in the development of coronary artery disease in addition to other diseases (14). As mentioned previously, reactive oxygen radicals, including superoxide and hydrogen peroxide are produced secondary to auto-oxidation during the metabolism of Hcy. These radicals cause endothelial damage (15). Besides, these free oxygen radicals induce DNA damage, mutations, sibling chromatid exchanges and chromosomal aberrations. Malignant transformations have been shown in cells to which free radicals produced by neutrophils, H_2O_2, or systems that produce free radicals free of cells (xanthine oxidase/hypoxanthine) are transferred. In order to protect the body from these deleterious effects of reactive oxygen radicals, antioxidant systems intervene. One of these systems is catalase, which has an enzymatic structure. Catalase manifests its antioxidant effects via splitting H_2O_2 to oxygen and water. Starting from this point, studies have been conducted in order to explain the relationship of Hcy and free oxygen radicals with carcinogenesis. Shinji Oikama et al (16) and Lily et al (17) have reported that oxidative DNA damage takes place through similar pathways, and leads to mutations of genes such as p53 and ras, finally leading to carcinogenesis. Although the Hcy levels in our study were not that high (such as 100 m), lower Hcy levels may possibly cause DNA damage in vivo.

Hcy levels are known to be high in patients receiving methotrexate. However, Refsum et al (18) reported in 1991 that in a study conducted in 12 pediatric patients with acute lymphoblastic leukemia, the Hcy levels following methotrexate treatment were higher than those before methotrexate or any other treatment, and this was said to be a new discovery. During follow-ups, they observed that the Hcy levels in the circulation decreased considerably following cytotoxic treatment. This study was the first to report that Hcy levels could rise in cancer patients without antifolate drug administration. In this report, emphasis was put on the fact that Hcy levels were indicative of the total malignant cell load.

In patients with folate deficiency, high Hcy levels are more significant risk factors for cancer. Folic acid has a critical role in the re-methylation step of the pathway in which Hcy is converted to methionine. Folic acid deficiency of any etiology leads to disruption of the re-methylation step, and subsequently to increased Hcy levels (19).

Likewise, Glynn et al (20) and Martinez et al (21) have indicated at this point in their studies reporting that subjects with folic acid deficiency may have increased risk of colon cancer. Therefore it has been proposed in these reports that folate may have a protective role against colon cancer.

Apart from high Hcy levels being related to insufficient folic acid ingestion, relationships have also been found between genetic polymorphisms of the 5,10-methylene-tetrahydrofolate reductase (MTHFR) enzyme of the re-methylation step, colon neoplasias and high Hcy levels. 5-Methyl tetrahydrofolate (THF) is the major form of folate in serum and acts as the methyl donor in re-methylation of Hcy to methionine. 5-Methyl THF is first obtained via conversion to tetrahydrofolate, and then to methylenetetrahydrofolate. Here, the 5,10-methylene-tetrahydrofolate reductase enzyme has the critical role of catalyzing the conversion of methylene THF to 5-methyl THF. Genetic polymorphisms of this enzyme reduce the synthesis of 5-methyl THF. Therefore, Hcy cannot be converted to methionine, subsequently leading to high Hcy levels that are associated with cancer. However, alterations of this enzyme may take place through environmental influences (22).

A point that attracts attention here is that 5-methyl THF provides methyl groups for neurotransmitters, proteins, nucleic acids and phospholipids. In previous studies, it has been demonstrated that DNA methylation has significant roles in gene stability and repair. In the early stages of neoplastic cell development, disturbances of DNA methylation such as local hypermethylation or hypomethylation may be observed. When compared with normal tissues, there is global hypomethylation in tumors. If DNA repair mechanisms do not function properly, the rate of mutations increases and chromosomal stability is impaired. As a result, a neoplastic event may be initiated or accelerated. Due to genetic polymorphisms of the enzyme 5,10-methylene-THF reductase, reduced 5-methyl THF levels may lead to unavailability of the methyl groups required for DNA methylation. Hence, DNA is hypomethylated. This situation explains the relationship between the afore-mentioned enzyme and colorectal-folate-Hcy levels (22, 23).

Folate has a critical role in de novo synthesis of purines and thymidine, and in the inter-conversion of amino acids. As a result of folate deficiency, cell proliferation is inhibited; the cell cycle is disrupted,

followed by genetic damage and eventual cell death. In a study conducted by Chern et al (24), who have explained the mechanisms by which folic acid deficiency causes these situations, the relationship between oxidative stress secondary to folic acid deficiency and apoptosis was investigated, and a comparison between cultures of Hep G 2 cells deficient of folic acid and cells in the control group was made. Folate deficiency was found to be capable of activating a redox-sensitive transcription factor, NF-kappaB, which is crucial in the control of a reactive oxygen species-mediated apoptosis. The Hcy levels increase in of folic acid deficient states, resulting in increased hydrogen peroxide levels. Thus, the increased activation of NF-kappaB secondary to overproduction of hydrogen peroxide causes DNA damage and eventually, apoptosis.

Beside the fact that high Hcy levels are risk factors for cancer, this situation has been proposed to be used as a tumor marker for rapidly proliferating tumor cells. In a study conducted with this purpose by Chien-Feng-Sun et al (19), cell cultures of human fibroblasts and various tumors (lung, breast, prostate, colon and neuroblastoma) were prepared. When normal human cell cultures were compared with cancerous cell cultures, the cancer cells were found to secrete more Hcy. However, when the amounts of Hcy secretion per cellular unit were compared, the Hcy secretion by cancer cells was found to be only mildly increased. In the second phase of the same study, the Hcy levels in normal and cancer cell cultures were compared with the levels of specific tumor markers of correlated tumors (CA 125 for ovarian cancers, CA 15-3 for breast cancer, CA 19-9 for pancreas cancer, and CEA for colonic cancer). Within a 7-day period, the increases in the levels of Hcy and tumor markers paralleled each other. At the end of the 7^{th} day, when the cells in the cultures began to die, the Hcy concentrations decreased while the tumor markers continued to increase. Accordingly, it has been concluded that Hcy is solely secreted by live cells, and therefore may be a helpful tumor marker reflecting tumor activity. This study, unlike previous studies, raises the suggestion that Hcy may be a result, rather than a cause of cancer.

In the study we conducted, the Hcy levels in esophageal, gastric and colonic cancer patients were significantly higher than those in controls. These data are compliant with those in literature. However, the initial correlation between Hcy and tumor markers in normal and cancer cell cultures as reported in literature was not observed in our study. This may possibly be related to the fact that the previous results were obtained in vitro while our study was conducted in vivo. Besides, the statistically significant increment in the Hcy levels in cancer cases during the period that the increment in tumor markers were insignificant, supports the hypothesis that Hcy might serve as a useful tumor marker.

Lily et al (17) have reported similar results, and stressed that Hcy may serve as a good tumor marker in the monitorization of patients receiving treatment as it does not only reflect the proliferation but might also reflect the death of tumor cells. Moreover, it has been proposed that Hcy might be an early tumor marker for carcinogenesis and a sensitive tumor marker for screening of recurrences.

The general approach we achieved during review of the literature was that the answer to whether hyperhomocysteinemia was a cause or a result of cancers is not clear. Further research is required in order to find out the correct answer to this question. However, it appears relevant to use Hcy as a tumor marker until the answer to this question is found, as the statistically significant increments in Hcy levels versus insignificant increments in the specific tumor markers in esophageal, gastric and colonic cancer patients reflected in our results support our hypothesis. In conclusion, the significantly higher Hcy levels in patients of esophageal cancer found in this study proving the relationship between GIS cancers, as important causes of morbidity and mortality, and Hcy raises the suggestion that Hcy might serve as a tumor marker. The statistically significant increases in Hcy levels versus the insignificant increments in tumor markers in patients with esophageal cancer further support our conclusion. Is hyperhomocysteinemia a cause or a result of cancer? We propose further studies to be conducted in order to explain this discrepancy.

References

1. Kadayifci A, Benekli M, Savas C. Tumor Belirleyicileri. Turkiye Tip Dergisi 1994; 1: 273-284.
2. Hergenc G, Sahin H. Kanser tanı ve prognozunda tümör belirteçlerinin önemi. Sendrom 1994; 4: 50-5.
3. Maxwell P. Carcinoembriyonic antigen: Cell adhesion molecule and useful diagnostic marker. Br J Biomed Sci 1999; 56: 209-14.
4. Prasad K. Homocystein a risk factor for cardiovaskular disease. Int J Angiology 1999; 8:

76-86.
5. Ramussen K, Moller J. Total homocysteine measurament in clinical practice. Ann Clin Biochem 2000; 37 (5): 627-648.
6. Ueland PM, Refsum H. Plasma homosysteine a risk factor for vascular disease; plasma levels in health.disease and drug therapy. J Lab Clin Med 1989; 114: 473-501.
7. Finkelstein JD. Homocysteine: A history in proress. Nutrition Rewiews 2000; 58 (7): 193-204.
8. Araki A, Sako Y. Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection. 1987;422:43-52.
9. Tasan G, Avsar E. Esophageal tumors. In Özden A, Şahin B, Yılmaz U, Soykan İ. Eds. Gastroenteroloji. Ankara: Türk Gastroenteroloji Vakfı, 2002: 61-66.
10. Fiscbach W, Ritter P. How are tumor markers used in diagnosis and in after-care of gastrointestinal cancers ? Results of nation-wide German survey. Med Clin 1992; 87: 576-9
11. Stamm EB, Reynolds RD. Plasma total homocystine may not be the most appropriate index for cardiovascular disease risk. J Nutr 1999; 129:1927-1930.
12. Schneede J, Refsum H, Ueland PM. Biological and enviromental determinants of plasma homocysteine. Semin Thromb Hemost 2000; 26: 263-79.
13. Welch GN, Loscalzo J. Homocysteine and atherothrombosis. New Eng J Med 1998; 338: 1042-1050.
14. Nicoletta B, Maria GA, Samanta M, et al. Genetic polymorphisms in folate and homocysteine metabolism as risk factors for DNA damage. Eur J Human Genetics 2003; 11: 671-678.
15. Loscalzo J. The oxidant stress of hyperhomocysteinaemia. J Clin Invest 1996; 98: 5-7.
16. Shinji O, Katsuhiko M, Shousuke K. Oxidative damage to cellular and isolated DNA by homocysteine: implications for carcinogenesis. Oncogene 2003; 22: 3530-3538
17. Lily LW, James TW. Hyperhomocysteinemia is a risk factor for cancer and a new potential tumor marker. Clinica Chimica Acta 2002; 322: 21-28.
18. Refsum H, Wesenberb F, Ueland PM. Plasma homocysteine in children with acute lymphoblastic leukemia: changes during a chemotherapeutic regimen including methotrexate. Cancer Res 1991; 51: 828-35
19. Chien-Feng S, Thomas RH, Tsu-Lan W, et al. Serum total homocysteine increases with the rapid proliferation rate of tumor cells and decline upon cell death: a potential new tumor marker. Clinica Chimica Acta 2002; 321: 55-62.
20. Glynn SA, Albanes D, Pietinenn P, et al. Colorectal cancer and folate status: a nested case-control study among male smokers. Cancer Epi Bio Prev 1996; 5: 487-494
21. Martinez J, Stampfer MJ, Giovannucci E, et al. Methylenetetrahydrofolate reductase polymorphizm, dietary interactions, and risk of colorectal cancer. Cancer Res 1997; 57: 1098-1102
22. Miguel A.M, Jose LU, Maria IA. Role of homocysteine in cell metabolism. Eur J Biochem (2001); 268: 3871-3882
23. Maria FP, Sonia A, Mario FF, et al. Germ-Line Variants in Methyl-Group Metabolism Genes and Susceptibility to DNA methylation in Normal Tissues and Human Primary Tumors. Cancer Research 2002; 62: 4519-4524.
24. Chern CL, Huang RF, Chen YH, et al. Folate deficiency-induced oxidative stres and apoptosis are mediated via homocysteine-dependent overproduction of hydrogen peroxide and enhanced activation of NF-kappaB in human HepG2 cells. Biomed Pharmacother 2001; 55 (8): 434-42.