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The effect of some trace elements on the expression of telomerase gene in lung cancer

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ABSTRACT

Lung cancer is a disorder that begins due to genetic and epigenetic changes. These changes cause the activation of oncogenes and the inactivation of tumor suppressor genes. Various factors influence the expression of these genes. In this research, we investigated the relationship between the number of trace elements zinc and copper and the ratio of these two in serum with the expression of the telomerase enzyme gene in lung cancer. For this purpose, we included 50 people with lung cancer in the study as the case group and 20 patients with non-tumor lung diseases as the control group. The TRAP assay method measured the telomerase activity in biopsy samples of lung tumor tissue. Also, serum copper and zinc were measured by atomic absorption spectrometry. The results showed that the mean serum concentration of copper and the ratio of copper to zinc in patients were significantly higher than in the controls (120.8 ± 5.7 versus 107.2 ± 6.5µg/dL)(P<0.05). However, there is no significant difference in the mean serum concentration of zinc between the two groups ($p > 0.05$). The patients' mean ratio of copper to zinc is significantly higher than the control group (1.6 ± 0.4 versus 1.1 ± 0.2)($p < 0.05$). The average level of telomerase enzyme activity of patients showed a significant difference from the control group 32.8 ± 16.1 vs. zero percent) ($p < 0.01$). There is a direct and significant correlation between the serum level of copper and the level of telomerase enzyme activity in patients ($r = 0.36$ and $p < 0.05$). A positive correlation was observed between total serum copper concentration and the increasing age of patients ($r = 0.39$ and $p < 0.01$). The correlation between the ratio of copper to zinc and the serum copper concentration of the patients was positive and significant ($r = 0.36$ and $p < 0.05$), but the correlation between the amount of serum zinc and the ratio of copper to zinc was negative and significant ($r = -0/ 72$ and $p < 0.01$). The average copper serum concentration of people with small cell carcinoma (123.7 ± 2.8 µg/dL) compared to non-small cell carcinoma (117.6 ± 4.8 µg/dL) is high and significant (P< 0.05). Patients with small cell carcinoma had a high mean telomerase concentration (112 ± 0.57%) and significantly compared to non-small cell carcinomas (6.4 ± 2.5%)($P < 0.05$). This situation regarding the zinc element and the ratio of copper to zinc in non-small cell carcinomas compared to small cell carcinomas were evaluated as non-significant (P>0.05). Based on the obtained results, it can be assumed that determining the amount of zinc and copper and the telomerase enzyme activity in lung cancer can have a biological role in the initiation and progression of tumor tissue, which requires more studies.

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Introduction

One of the essential characteristics of cancer cells compared to body (non-cancerous) cells is their very high and unlimited replication ability (1). The most critical controllers of cell replication are part of the telomere chromosome structure. DNA polymerases cannot wholly replicate the 5′ ends of linear chromosomes (2). A part (50-100bp) of the 5′ ends is lost in each cell division because telomeres play an important role in controlling the speed of cell division and the aging process when the process of telomere shortening to a dangerous level (removal coding sequences) will cause natural cell death (senescence) (3). Telomere length is fixed due to the activity of an enzyme called telomerase (4). This enzyme is a Reverse Transcriptase that, by adding repetitive sequences isolated from the 5′ ends of chromosomes, causes the replacement of lost pieces of DNA during each cell division. It stops the natural cell death process by preventing the shortening of telomeres (2, 5). Telomerase is active in almost all cancer cells. Research shows that telomerase activity is related to the stages of cancer progression. Therefore, the measurement of telomerase activity can be a reliable indicator in cancer diagnosis and in determining the stages of its progress (6). In addition to tumor cells, specific normal cells such as stem cells and germ line cells have unlimited proliferation power due to the stability of the telomeres of these cells (7).

Interestingly, telomerase is reactivated in a wide range of cancer cells. Telomerase activity is the most common molecular marker for identifying human cancer and can be detected in 85% of all tumors (8). At the same time, most healthy tissues show low levels of telomerase activity or lack telomerase activity (4). Various studies have shown

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that measuring telomerase activity is effective in diagnosing early stages of cancer that other methods cannot detect. Therefore, telomerase can be considered a primary and independent marker of cancer (7, 9). In some cases (notably neuroblastoma and gastric and breast tumors), high levels of telomerase activity are associated with poor prognosis, indicating that telomerase activity can be used as a predictive marker (9). Therefore, despite proving the high level of telomerase activity in tumor cells of different origins and measuring the activity level of this enzyme in lung cancer, which has been done scattered, so far, no study indicates the level of activity of this enzyme in normal human cells and cells (10). No cancer, especially lung cancer, has been observed, so this study was conducted to determine the activity level of telomerase enzyme in the control group (non-cancerous lung diseases) and case (lung cancer patients) (11).

The biological role of essential trace elements, especially the changes in copper and zinc levels in various cancers, has been the focus of different research teams in recent years. Zinc, one of the essential trace elements in the body, is an important structural component of more than 200 metalloenzymes involved in the pathways of cell proliferation, DNA repair, antioxidants against free radicals (factors involved in causing cancer) such as copper superoxide dismutase enzyme, etc (10, 12). Also, the APA protein carrying zinc cofactor binds to the promoter of the telomerase enzyme gene (responsible for the unlimited proliferation of cancer cells) and reduces its expression (13). As another rare element of the body, copper is part of the structure of some proteins, such as plasma ceruloplasmin, vascular endothelial growth factor, and transcription factors. These three factors have increased along with copper in the serum of some people with malignant cancers (14). Many reports indicate that cancer cells have a very high concentration of copper ions due to their ability to absorb copper ions from the non-ceruloplasmin part of the plasma. This element plays a vital role in tumor growth in angiogenesis through the amine oxidase pathway dependent on copper ions (15). Since there is a lot of competition between zinc and copper elements to enter tissue cells, the changes in copper to zinc levels in the serum are also significant. However, different results have been reported about the benefit of using the changes of these two elements and the ratio of copper to copper in the body in different cancers, especially lung cancer, from the point of view of cancer diagnosis and prognosis. Therefore, according to most of the previous reports that the level of telomerase activity increases in many types of cancers (13, 16), this study aims to evaluate the relationship between the level of telomerase activity in non-cancerous lung diseases and the level of telomerase activity in samples obtained from lung cancer patients. Also, the relationship between the serum concentration of zinc and copper elements and the activity of the telomerase enzyme was investigated.

Materials and Methods

In this case-control study, 50 patients with lung cancer were included in the case group, and 20 patients with non-tumoral lung diseases (pulmonary infection, inflammation, etc.) were included in the control group. The Ethics Committee approved the study protocol of the Tuberculosis and Pulmonary Research Center, and written consent was obtained from all patients. Based on cytology and histopathology studies of biopsy samples, patients were divided into two main groups small-cell carcinoma and non-small cell carcinoma (adenocarcinoma, squamous cell, and large cell) lung cancer. We took 5 ml of blood from all the studied subjects, and its serum was separated by centrifugation at 3000 rpm for 10 minutes at 4 degrees Celsius. We kept biopsy samples of patients along with all serum samples at -70 degrees Celsius until the relevant analyses.

Tumor tissue cytosol extraction

In order to extract tumor cytosol, first, tumor tissue samples were powdered in liquid nitrogen using the Tissue hammering device designed in the laboratory. Then, 200 microliters of cell lysis buffer (10mM Tris-HCl, 1mM magnesium chloride, 0.1mM benzamidine, 5mM beta-mercaptoethanol, 0.5% chaps, 10% glycerol, and 1mM EDTA) was added and spun for 20 minutes at 1500 rpm was centrifuged. The supernatant was slowly transferred to a new micro-tube (17).

Measurement on the surface

First, the serum samples were diluted with deionized water at a ratio of 1 to 5, and the amount of zinc and copper was measured by Atomic Absorption Spectroscopy Analytical CTA 2000 Chemtech. The basis of this device is the excitation of ions by flame or heat, the electrons are excited to a higher layer, and upon returning to the initial state, UV rays are emitted, which are recorded at a specific wavelength for each ion by the stability section of the device. For the quality control of this test, standard reference materials were used.

TRAP Assay

To measure telomerase enzyme activity, the TRAP Assay method based on two polymerase chain reaction techniques and ELISA based on Holt's method was used (19). To optimize TRAP-PCR conditions, the concentration of the reagent for a volume of 50 microliters, including 10micrograms of extracted protein sample containing telomerase enzyme, 5microliters of dNTP with a concentration of 10 mM, 60 nanograms of primer, 5 microliters of a standard containing the sample and 16 microliters of double distilled sterile DNA was prepared.

It should be mentioned that Taq Polymerase plays the role of telomerase enzyme in this reaction. After preparing the reagents, the necessary program to perform PCR was set up in the thermocycler model of Eppendorf Company. In this program, one round for 30 minutes at a temperature of 30 degrees Celsius to prepare the telomerase enzyme to start its activity, and then one round for 1 minute to become single-stranded at a temperature of 94 degrees Celsius, 27 rounds including the steps related to primer binding for 30 seconds, which The binding temperature for the primer in the kit is specified as 30 degrees Celsius, the synthesis of telomeric sequences was set for 30 seconds at 59 degrees Celsius and the unwinding of the strand was set for 30 seconds at 94 degrees Celsius on a thermocycler.

After PCR, the ELISA method was used to measure telomerase enzyme activity. In the first step, 3microliters of the multiplication sample were moved and settled on the plate. After incubating the plate for 2 hours at room temperature, it was washed three times using the appropri-

ate buffer. Then the antibody labeled with biotin was added to the resulting complex. The corresponding antibody was conjugated with peroxidase. By adding the substrate solution containing 3,3,5,5-tetramethyl benzene (TMB), a color reaction took place. The amount of color created at a wavelength of 450 nm was read in an ELISA reader. By placing the samples' absorbed values in the standard curve, we identified the relative activity of telomerase enzyme in unknown samples.

Statistical analysis

An Independent T-test was used to analyze the data. Statistical significance ($p<0.05$) was considered for all two-tailed statistical tests. All statistical tests were performed using SPSS 11.5 statistical software.

Results

In this study, 50 people (38 men and 12 women) with lung cancer with an average age (37.9 ± 88.63, age range of 55 to 73 years) who were previously confirmed by invasive bronchoscopic and histopathological methods of lung cancer were investigated. Regarding histopathology, 18 people had small cell carcinoma, and 32 had non-small cell carcinoma. Determination of zinc and copper serum levels along with telomerase gene expression indirectly measuring telomerase enzyme activity (at the level of tumoral and non-tumoral lung tissues). It showed that the average copper serum concentration and copper-to-zinc ratio in people with lung cancer are significantly higher than in the control group ($p<0.05$). However, there is no significant difference between the average concentration of zinc serum in patients and controls ($P>0.05$). The average activity level of telomerase enzyme in the patient group showed a significant difference from the control group ($P<0.01$) (Table 1).

The serum levels of zinc and copper, along with the activity of telomerase enzyme in the tumoral tissue of patients with small and non-small cell carcinoma, showed that the mean serum concentration of copper in patients with non-small cell carcinoma was high and significant compared to small cell carcinoma ($P < 0.05$). Patients with small cell carcinoma had high and significant mean telomerase concentrations compared to non-small cell carcinomas ($P<0.05$). This situation regarding the zinc element and also the ratio of copper to zinc in non-small cell carcinoma compared to small cell carcinoma was evaluated as non-significant ($P>.05$) (Table 2).

In the study of the correlation and dispersion of the average values of the measured indices of copper and zinc and the activity of the telomerase enzyme in lung cancer samples, it was shown that there is a direct and significant correlation ($r = 0.36$ and $p < 0.05$) between the serum level of copper with level of telomerase enzyme activity in patients (Figure1).

Also, a positive correlation ($r = 0.39$ and $p < 0.01$) was observed between total serum copper concentration and the increasing age of patients (Figure 2). The correlation

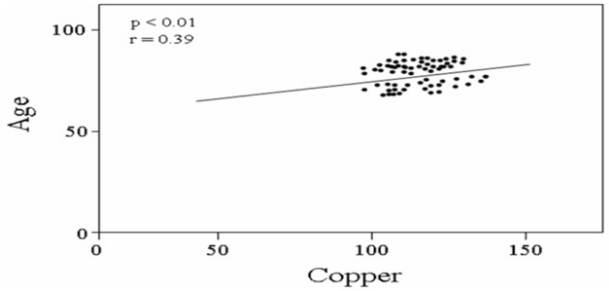

Figure 2. Correlation diagram between serum copper concentration and age of patients.

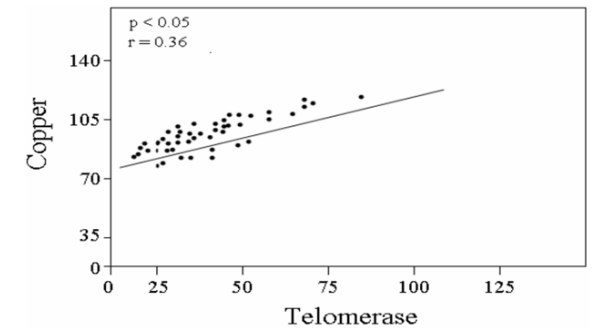

Figure 1. Correlation diagram between copper serum concentration and telomerase enzyme activity.

Table 1. Age characteristics and serum levels of copper, zinc, and the ratio of copper to zinc along with the telomerase enzyme activity in control and patient groups.

Variable	Control group Mean ± Standard deviation	Case group Mean ± Standard deviation
Age (year)	68.3 ± 3.8	64.9 ± 1.4
Copper (µg/dL)	107.2 ± 6.5	120.8 ± 5.7
Zinc (µg/dL)	87.0 ± 4.1	38.8 ± 3.8
Copper/Zinc	1.1 ± 0.2	1.6 ± 0.4
Telomerase (%)	0	32.8 ± 16.1

Table 2. Comparison of serum levels of copper, zinc and the ratio of copper to zinc along with the activity of telomerase enzyme in small and non-small cell carcinoma of the lung.

Variable	Small cell carcinoma Mean ± Standard deviation	Non-small cell carcinoma Mean ± Standard deviation	P-value
Age (year)	61.22 ± 10.3	64.0 ± 7.8	Non-Significant
Copper (µg/dL)	123.7 ± 2.8	1117.6 ± 4.8	<0.05
Zinc (µg/dL)	82.7 ± 1.4	81.2 ± 2.9	Non-Significant
Copper/Zinc	1.5 ± 0.02	1.4 ± 0.5	Non-Significant
Telomerase (%)	112.0 ± 0.57	6.4 ± 2.5	<0.05

between the ratio of copper to zinc with the serum concentration of copper in the case group was positive and significant ($r = 0.36$ and $p < 0.05$), but the correlation between the amount of serum zinc and the ratio of copper to zinc was calculated to be negative and significant ($p < 0.01$ and $r = -0.72$).

Discussion

Cancer is a widespread problem in all societies of the world, and a set of factors, including tumor markers and essential elements such as zinc and copper, play a role in its initiation and progression (14, 18). Lung cancer is one of the most common types of cancer (19). In this research, due to the importance of this type of cancer, we investigated the activity of telomerase enzyme at the tumor tissue level, as well as the serum levels of zinc and copper as factors involved in its onset and progression in lung patients. Telomerase is inactive in most somatic cells, while it is active in more than 90% of cancer cells. In this study, it was observed that the level of telomerase activity in lung cancer patients is high (32.8 ± 16.1%), while no activity was observed in the control group (0%). This activity difference between cancerous and non-cancerous tissues was statistically significant, similar to the results of Mokbel et al. (20). In this way, measuring telomerase enzyme activity in lung cancer tissues can help diagnose it. It indicates a relationship between telomerase activity and disease prognosis (20). Regarding the role of metals such as copper and zinc and the ratio of these two elements in connection with the initiation and progression of tumor cells, many investigations have indicated the changes of these two elements in the serum levels of cancer patients. In our study, it was also observed that the copper concentration and the copper-to-zinc ratio in the case group are higher than in the control group, and this difference is statistically high ($P<0.05$).

The zinc concentration in the case group was lower than in the control group, but no significant difference was observed between them. On the other hand, the correlation between the serum concentration of copper and zinc with the ratio of copper to zinc in the patients was calculated to be significantly positive and significantly negative, respectively, which is in agreement with the results of Zowczak et al. (21), Piccinini et al. (22).

In their study on 84 patients with lung cancer, Oyama et al. (23) showed that the serum concentration of copper and the ratio of serum copper in the patients were significantly higher compared to the control group. In the study of this group in 1994, also on 162 patients with lung cancer, they calculated the correlation between the serum concentration of copper and zinc with the ratio of copper to copper in the patients, respectively, significant positive and significant negative (24). Also, Zowczak et al.'s studies on patients with lung, breast, gastrointestinal, ovarian, and cervical cancers showed an increase in the amount of copper and the ratio of copper to zinc compared to the control group (21, 25). The decreasing changes in zinc elements were seen only in the initial stages of patients with gastrointestinal cancer. In the study he conducted on patients with lung cancer, Piccinini et al. (22) observed a significant decrease in the amount of zinc in lung cancer ($P<0.05$). Increasing the copper-to-zinc ratio probably increases the new veins' production process. Zinc also participates in the structure of protein antioxidants, called metallogeny and glutathione peroxidase, which reduces free radicals and copper entry into the tissues (26). As a result, mild copper deficiency in the tissue will result in the lack of growth of tumor cells. In our study, it was observed that there is a direct and significant correlation between the serum levels of copper and the ratio of copper to zinc with the activity of the telomerase enzyme in lung cancer patients ($r = 0.36$ and $p < 0.05$), however, there is a correlation between the serum concentration of zinc. It was not significant with the activity of the telomerase enzyme, which is similar to the results of Nemoto et al. (27). In their in vitro studies involving NRC2 cells, Nemoto et al. (27) showed that the changes in the concentration of zinc and copper elements in the initial stages of cultivation of these cells had a significant positive correlation with the activity of the telomerase enzyme. Of course, these changes after the first 6 hours were significant only for copper ion and copper to zinc ratio with positive telomerase enzyme activity. The reason for this problem can be justified as the effect of zinc element on increasing the activity of telomerase enzyme is indirectly through the expression of early response genes, and by reducing the expression of these genes, the activity of telomerase enzyme returns to its original state (28). However, copper's role in increasing this enzyme's activity is still unknown (29). Of course, the validity of the above-mentioned cases can be proven through in vitro studies with the effect of zinc and copper elements on the expression of the telomerase enzyme's TERT gene and other genes expressed in this process. In this study, it was observed that the average concentration of copper in the serum of people with non-small cell carcinoma compared to small cell carcinoma is high and significant ($P<0.05$), which has been confirmed by several studies and as a diagnostic marker. It is recommended for both types of cancer (30).

Patients with small cell carcinoma had a high and significant mean concentration of telomerase compared to non-small cell carcinomas ($P<0.05$). However, no material was found to confirm this finding, which requires further investigation. In summary, according to the results of this study, changes in the serum and tissue levels of the rare elements zinc and copper in the biological activities of cancer cells, primarily through the expression of the telomerase enzyme gene, probably play an essential role, and this requires further studies.

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