مقاله تحقیقی

اثرات مهاری روی، مس و سلینیوم بر فعالیت تلومراز در بافت های توموری پستان و رده سلولی سرطان پستان T47D

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چکیده

اهداف: مطالعه حاضر برای بررسی اثرات عناصر کمیاب روی (Zn)، مس (Cu) و سلینیوم (Se) بر تومورزایی در سرطان پستان انجام گرفت. اثرات مهاری روی (Zn)، مس (Cu) و سلینیوم (Se) بر فعالیت تلومراز در بافت سرطان پستان انسانی و رده سلولهای سرطان پستان T47D انجام گرفت.

روش ها: نمونه های توموری از ٢٤ زن دارای توده خوش خیم و ٣٢ زن مبتلا به سرطان پستان (داکتال کارسینوما، لوبولار کارسینوما) در طول جراحی جمع آوری گردید. نمونه خون وریدی جهت تعیین سطح عناصر کمیاب گرفته شد. رده سلول سرطانی پستان پس از کشت با عناصر کمیاب تمیار شد سپس، هم در عصاره استخراج شده از بافتهای توموری و هم در رده سلولی تیمار شده، فعالیت تلومراز با روش TRAP اندازه گیری شد.

یافته ها: نتایج نشان داد که یک ارتباط معنی داری بین سطح سرمی و سطح بافتی مس، سلینیوم و روی/مس در بیماران و افراد کنترل وجود دارد ($P< 0.001$). شش ساعت بعد از تیمار با ZnSO4 (١٠٠ میکرومول بر لیتر) و CuSO4 (١٠ میکرومول بر لیتر)، فعالیت تلومراز در رده سلول سرطانی پستان T47D بطور معنی داری افزایش یافت اما بعد از تیمار با سلینیوم-ال-متیونین با غلظت ١٠ و ٣٠ میکرومول بر لیتر فعالیت تلومراز بطور معنی دار کاهش می یابد بطوری که فعالیت تلومراز در رده سلول سرطانی پستان T47D ٢٤ ساعت بعد از تیمار به ترتیب ٠/٩٣٪ و ٠/٦٠٪ و ٤٨ ساعت بعد به ترتیب ٠/٧٦ ٪و ٠/١٢٪ در مقایسه با نمونه کنترل (٤٩/٢٪) بود. سطح سرمی روی و مس در بین بیماران متغیر بود.

نتیجه گیری: بطور کلی ارتباط معنی داری بین سطح عناصر کمیاب و میزان فعالیت تلومراز می تواند بعنوان یک مارکر پیش بینی و تشخیصی در سرطان پستان در نظر گرفته شود.

کلمات کلیدی: *سرطان پستان، روی، مس، سلینیوم، تلومراز*

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ORIGINAL ARTICLE

Inhibitory Effect Of Zinc, Copper And Selenium On Telomerase Activity In Tumor Tissues And T47D Breast Cancer Cell Line

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A B S T R A C T

Background: The present study was performed to investigate the effects of trace elements particularity Se, Zn and Cu on tumor genesis in breast cancer.
Methods: The inhibitory effect of Se, Zn and Cu, on telomerase activity was analyzed in human breast tumor tissues and breast cancer (T47D) cells. Tissue specimens from 24 women with benign breast disease and 32 women with breast cancer specimens (ductal carcinoma, lobular carcinoma) were collected during surgery. In addition venous blood samples were obtained for assessing the trace elements. T47D cell line was cultured and treated with trace elements. Telomerase activity then was measured with TRAP assay in cell line and tissue extracts.
Results: There was a significant difference between tissue and serum levels of Cu, Se and the ratio of Cu/Zn in patients and controls (P<0.001). After treating with 100 µm/L Zn So4, 10 µm /L Cu So4 for 6 hours, telomerase activity of T47D cells was markedly increased. But after treating with 10, and 30 µm /L selenium-L- methionin, telomerase activity was markedly inhibited. Telomerase activity of T47D cells for 24 hours were 0.93, 0.60 and for 48 hours were 0.76, 0.12 respectively (control 49.2%). There were variations in serum level of Zn and Cu in breast cancer patients.
Conclusion: Association between trace elements level and telomerase activity level can be exploited as prognostic and diagnostic marker for breast cancer.

Keywords: *Breast cancer, Zinc, Copper, Selenium, Telomerase.*

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INTRODUCTION

Breast cancer is the most frequent cancer among women. Breast cancer accounts for %22 of cancers among females worldwide and %9.6 percent among Iranian women (1). Several factors such as genetics, dietary, environmental factors, hormone activity or a combination have been identified, to understood this disparity (2). Studies have found that telomerase and trace elements particularity Se, Zn and Cu could have a role in tumor genesis (3,4).

Telomerase, a specialized RNA dependent DNA polymerase which synthesizes telomeric repeats, is present in most cancer cell lines and in certain germ line and stem cells, but not normal cells (5). In most cases it is molecular base of unlimited proliferation. Human telomerase is composed of two essential components: an integral RNA (hTR), which provides a template for the synthesis of telomeric repeats, and a protein subunit (hTERT) which provides catalytic activity (5, 6). The RNA component is present in all cells while the expression of hTERT is confined to cells that have telomerase activity (6, 7). This means that hTR expression is not recognized as a rate-limiting factor for enzyme activity (8, 9). Cloning of telomerase catalytic subunit gene (hTERT) showed a direct correlation between expression of this gene and telomerase activity (6, 7).

Experimental and epidemiological studies on the relationship between trace elements and carcinogenesis and comparing these in serum, tumor tissue and normal tissue suggest that selenium (Se) inhibits carcinogenesis (10). Selenium is an antioxidant which protects cells from DNA oxidative damage by scavenging free radicals in breast cancer-causing epithelial cells (11). The relationship between selenium status and risk of cancer demonstrated that a decrease in Se concentration is related with increased telomerase activity and stimulates apoptotic process (12). However, a prospective study demonstrated that difference in Se levels between tumoral and control samples were not significant (13).

Zinc (Zn) is an essential mineral element that is integral part of many proteins and transcription factors that regulate key cellular functions such as the response to oxidative stress, DNA replication, DNA damage repair, cell cycle progression, apoptosis and gene expression such as telomerase gene expression (14,1516). In addition, Zn is present in Zinc finger motifs such as MZF2 in steroid hormone receptors, APA1 and other regulatory proteins in gene expression (15, 17). Zn releases cytochrome C from mitochondrial membrane, causes thereby activation of caspases and initiation of apoptosis (12, 17). Final effect of Zn is decreasing the number and size of tumor cells (12).

Copper (Cu) is the other trace element with a role in more than 9 growth factors especially in VEGF. It can stimulate angiogenesis and cause tumor initiation and metastasis (18).

Studies have shown inconsistent results. Therefore the aim of this study was to evaluate the effects of trace elements including Zn, Cu and Se after treatment on telomerase activity in the T47D breast cancer cell line. Since telomerase activity can be used as a diagnostic and therapeutic marker the study also aimed to examine the effect of serum levels and tumor cytosol extract levels on telomerase activity in breast cancer patients and women with benign breast disease.

METHODS

Sample preparation

Tissue specimens from 24 women with histologically confirmed benign breast disease (fibro adenomas, fibrocystic disease and adenosis) and 32 breast cancer specimens (ductal carcinoma, lobular carcinoma) were collected during surgery at the department of surgery of

Imam Hospital, Tabriz University of Medical Sciences. The specimens were snap-frozen in liquid nitrogen immediately after surgical restriction, and were stored at -80°C until use. Blood samples were obtained and were their serum was isolated immediately.

Tumor cytosol extraction

Approximately 0.2 g of tissue from each tumor was quick frozen and pulverized manually with a hammer to a fine powder at -80 C. The cells were lysed for 30 minutes on ice with 1 ml of lyses buffer (50mmol/L Tris buffer, pH 8.0, containing 150 mmol/L NaCl, 1 mmol/ L phenelmetylsulfonyl fluoride, 10g/L Nonident NP-40 surfactant and 5 mmol/L EDTA) The lysates were centrifuged at 15000 g at 4° C for 30 minutes and the supernatants were assayed for the levels of Zn, Cu and Se by using atomic absorption spectrometry (AAS) and Graphite atomic absorption spectrometry. Blood samples were obtained and their serum was isolated immediately and levels of Zn, Cu and Se were measured using previous producers.

Cell culture and treatment

Cells of the T47-D breast cancer cell line (National Cell Bank of Iran) were grown in DMEM medium supplemented with 10% (v:v) heat-inactivated fetal bovine serum, penicillin G (80mg/ml), streptomycin (50 mg/ml) and at 37°C in a humidified atmosphere of 5% CO2 in incubator. When the cells were 70-80% confluent, they were washed once with PBS and after counting, 10^6 cell a liquated to each flask and exposed for different times (0, 6, 24 and 48 hr) in 5 mL DMEM medium with different concentrations of trace elements. After incubation, they were washed with PBS and harvested for performing TRAP (Telomeric Repeat Amplification Protocol) assay. Final doses of ZnSO4 in each flask were 0, 0.1 and 0.5 mmol, for CuSO4 were 0, 3 and 10µmol and for Se-L-methionin were 0, 10 and 30µmol as a single treatment, Cells in matching control group received same volume of the distilled water (distilled water was used as solvent of trace elements).

Extraction of total protein

Based on instructions of Telo TAGGG Telomerase PCR ELISA PLUS kit, Lysis reagent was added to the cell pellets and homogenized tumor tissue to lyses them completely. The samples were incubated on the ice for 30 minutes. The lysates were centrifuged at 16000g for 20 minutes at 4°C, and the supernatants were transferred to fresh tubes. 200µl of each extract was picked up for determining their protein concentration and performing TRAP assay, the remaining extract was immediately stored at -75°C.

Determination of protein concentration

Average protein content per sample was determined according to Bradford, using bovine serum albumin as the standard [19].

TRAP assay

Telomerase activity was determined using the Telo TAGGG Telomerase PCR ELISA plus detection kit. (Roche, Cat. Number: 2013789). This kit is a very sensitive in vitro assay system that uses PCR. In the first step, telomerase adds the telomeric repeats (TTAGGG) to the 3`-end of the biotin-labeled synthetic P1-TS (5'-AATCCGTCGAGCAGAGTT-3') primer. In the second step, these elongation products, as well as the internal standard (IS) included in the same reaction tube, are amplified by PCR using the primers P1-TS and the anchor-primer P2 (5'-(CCCTTA) 3CCCTAA-3'). For PCR starting the mixture was incubated at 95°C for 5 min before the addition of 5 U Taq polymerase and 0.1 mg P2 primer, and the elongated products were then amplified by PCR (30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s). PCR products resulting from telomerase-mediated elongation procedure, contained the telomerase-specific 6 nucleotide increments, while the IS generated 216 bp PCR product. In the third step, the PCR products are divided into aliquots, denatured and hybridized separately to digoxigenin -(DIG)-labeled detection probes, specific for the telomeric repeats (P3-T) and for the IS (P3-Std), respectively. The resulting products were immobilized via the biotin label to streptavidin-coated micro titer plate and then detected by using

the antibody against digoxigenin, which was conjugated to horseradish peroxidase (Anti- DIG-HRP) and the sensitive peroxidase substrate MB. TRAP assay is performed according to manufacturer's protocol.

RESULTS

The concentration of Zn, Cu and Se and the ratio of Cu/Zn have been measured in serum and tissue cytosolic extraction in patient and normal individual. The results showed the difference between serum levels of Zn, Cu and Se and the ratio of Cu/Zn between cases and controls (Table 1) .

Table 1. Serum concentrations of Zinc, Cupper, Selenium and Cu/Zn ratio in breast cancer patient and controls

	Mean ±SD		P value
	Control (n=50)	Case (n=50)	
Zn (mg/Lit)	1.07±0.35	0.97±0.19	0.119
Cu (mg/Lit)	1.09±0.20	1.47±0.47	0.002
Cu/Zn	1.12	1.52	0.000
Se	92.42±18.7	60.03±23.38	0.01

Mean serum levels of Zn, Cu and Se in breast cancer patients were 0.969 ±.19, 1.47±.48 mg/L and 60.04 ± 23.38 respectively. The mean concentrations of Zn, Cu and Se serum in women with benign breast diseases were 1.07±0.35, 1.09 ± 0.20 mg/L and 92.42±18.70 µg/L, respectively. There was a significant difference between Cu levels of patients and controls (P<0.001). In addition, the ratio of Cu/Zn in breast cancer patient and controls were 1.52 and 1.12, respectively. This difference was statistically significant (P<0.001) and also there was a significant difference between Se levels of patients and controls (P<0.001).

Mean tissue levels of Zn and Cu in breast cancer patients were 66.75±72.5 and 28.29±3.84µg/g respectively while the mean concentrations of tissues levels of Zn and Cu in controls were 26.7±28.5 and 21.02 ±6.08 µg/g respectively. There was a significant difference between Zn levels of patient and control (P<0.006). In addition, there was a significant difference between Cu levels of patients and controls (P<0.002). The ratios of Cu/Zn in patients and controls were 0.42 and 0.79, respectively. This difference was statistically significant (P<0.001). Also the mean concentrations of tissue levels of Se in patients and normal individual were 1.05±0.42µg/g and 0.51±0.22µg/g respectively. This difference was significant (P<0.01) (Table 2) .

Table 2. Tumor cytosol extracts concentrations of Zinc, Cupper, Selenium and Cu/Zn Ratio in breast cancer patient and controls.

	Mean± SD		P value
	Control(n=50)	Case(n=50)	
Zn(µg /g)	26.7±28. 5	66.75±72.5	P<0.006
Cu(µg/g)	21.02±6.08	28.29±3.84	P<0.002
Cu/Zn	0.42	0.79	P<0.001
Se(µg/g)	1.05±0.42	0.51±0.22	P<0.01

The analysis indicated that after treatment with 100 µm/L Zn So4 for 6 hours, telomerase activity of T47D cells was markedly increased (5.2 fold) but with 100 µm /L Zn So4 for 24 hours and 500 µm /L Zn So4 for 6, 24 hours, telomerase activity was 0.76, 0.39 and 0.12% respectively (in controld it was control 49.2%). After treatment with 10 µm /L Cu So4 for 6 hours, telomerase activity of T47D cells was increased 3.67 fold. Finally, after treatment with 10, 30 µm /L selenium-L- methionin, telomerase activity was markedly inhibited. Telomerase activity of T47D cells for 24 hours were 0.93, 0.60 and for 48 hours were 0.76, 0.12 respectively (in control was 49.2%) (Table 3)

Table 3. The inhibitory effects of different concentrations of Zinc, Cupper and Selenium on telomerase gene expression in T47D breast cancer Cell line.

Variable		Concentration	Incubation Time (hrs)	RTA (%)	*Fold increased*
$ZnSo_4$(mmol/L)	Control	0	0	49.2	1
		0.1	6	255.3	5.2
		0.5	6	37.8	0.76
		0.5	24	5.85	0.12
		0.1	24	19.2	0.39
$CuSo_4$(mmol/L)	Control	0	0	49.2	1
		3	6	50.3	1.02
		10	24	47	0.96
		10	24	180/7	3.67
		3	6	49/7	1.01
Se- L- mitionin	Control	0	0	49.2	1
		10	24	46	0.93
		30	48	5.84	0.12
		30	24	29.3	0.60
		10	48	37.5	0.76

DISCUSSION

There are variations in serum level of Zn and Cu in breast cancer patients (20). Zn is present in the structure of metalothionin and SOD, and then decreases free radicals. Zn has an important role on DNA polymerase and RNA polymerase functions. These roles cause fast growth of tumor tissue (17,21, 22). Cu is a cofactor for many enzymes and may be catalyzed ROS formation and lipid peroxidation (18, 23). The role of Zn, Cu and their ratio has been studied and demonstrated their direct relationship with initiation and progression of tumor (23). In the present study, there was no significant difference between Zn levels of patients and controls. This finding is in accordance with Yeou-Lih Huang study (20); but different from another study by Karunasinghe et al. (20, 23).

The study findings showed that Cu value in patient with breast cancer tumor was increased significantly in contrast to controls. This was compatible with the results obtained from other studies (12, 24).

As suggested Cu/Zn ratio in patient with breast cancer is important. The present study indicated that this ratio increased significantly and confirmed the results obtained by other investigators (12, 24). This means that increasing Cu/Zn ratio along with high MDA cause lipid peroxidation, disrupting antioxidant system and increasing ROS formation and resulting in high mutation and cancer formation (17,18 ,24). Cu is structural part of growth factors such as VEGF which play a role in angiogenesis. Therefore, increased level of Cu/Zn ratio causes new vascular formation (24,25); In the other hand, low level of Zn decreases competition between Cu and Zn absorption and causes increased absorption of Cu (24,25).

The findings from this study suggest that changes in the serum level of trace elements could be a prognostic and diagnostic marker in patients with breast cancer tumor. Se has a role in antioxidant system and can decrease ROS and cancer. Our study demonstrated that Se treatment of T47D breast cancer cells can decrease the progression of cancer cells (23,26). Se also can decrease telomerase activity and increase apoptotic process in lung cancer cells, resulting in limited progression of lung cancer cells (12). Other studies have shown that Se increases telomerase activity (27). However, a study has demonstrated that difference between Se level of tumor tissue and normal tissue was not significant (13). In the current study, cytosolic level of Zn in patients and individuals with benign diseases was statistically significant. Similar results were reported by other investigators (28, 29, 30). Difference in cytosolic level of Zn in patients and controls may be due to a defense system for saving Zn for using in growth and reproduction of cells (28). In contrast another study showed that Zn level was lower in cancerous gall-bladder compared to normal gall-bladder, although Cu level increased significantly (31). An earlier study showed that there were no significant changes in Cu level in patients and controls (32).

In the present work, the effect of trace elements on telomerase activity was studied, The results showed that 0.1 mmol ZnSO4 after 6 h treatment could increase 5.2 fold in telomerase activity. Probably Zn caused an increase in expression of proteins including Zn finger such as DNA polymerase binding protein, MYC, metalothionin and transcription factors. Therefore, increasing Zn level in the cell can cause increasing activity of transcription factors and finally, increasing expression of telomerase reverse transcriptase gene (hTERT). However, the results of a study is inconsistent with ours, because Zn induces P53 production and decreases telomerase activity by suppressing hTERT expression (33). When cells treated with 0.5 mmol ZnSO4 for 6 and 24 h, telomerase activity decreased 0.76 and 0.12 fold, respectively. Thus, Zn halts cell cycle by interfering with checkpoints (G1/S and G2/M checkpoints), inhibiting enzyme aconitase and Krebs cycle, releasing cytochrome C and cause apoptosis.

In this study, the results showed that 10 µmol CuSO4 after 6 h treatment could increase 3.67 fold in telomerase activity (33). It has been suggested that Cu is needed for angiogenesis, progression and metastasis by inducing growth of new vessels (25, 34).

Treating cells with Se-L-methionin in different dose and time demonstrated that telomerase activity decreased and the most decreasing (9.5 fold) occurred in 30 µmol after 48h. This finding is in accordance with finding from another study (12). A study also showed that low level of Se decreases DNA methylation , then DNA could not be repaired (35).

In conclusion, comparison of trace elements levels with telomerase activity in tumor tissue has demonstrated a paradoxical relationship. The findings showed that along with increasing Zn and Cu levels and decreasing Se level in the tissue, telomerase activity might increase. Comparison of trace elements levels in serum of benign and malignant breast tumor and comparison of their level in serum and tumor tissue and their correlation with telomerase activity suggest that their concentration can be used as prognostic and diagnostic marker. However, further studies are recommended.

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