STUDY OF CORRELATION BETWEEN SERUM ZINC LEVELS AND TELOMERASE ACTIVITY IN BLADDER CANCER PATIENTS

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SUMMARY: Telomerase is an active gene existing in almost all human cancerous cells. This enzyme is essential for immortality and recycling of these living cells, however it normally has not been expressed in the somatic cells. Role of zinc as a probably interfering microelement in telomerase gene expression and activity in human bladder cancer is the main subject of this study. Thirty three voided urine samples were gathered from patients, which were previously identified as bladder carcinoma cases using cytological methods. They had previously not given any treatment. Telomerase activity was determined using polymerase chain reaction, which refers to the telomeric repeat amplification protocol. In order to distinguish the fine signals, obtained the procedure was linked to an ELISA system (TRAP assay). The patients' grades and stages were determined independently through cystoscopy. Also, the serum zinc levels of these patients and control groups measured by atomic absorption spectrophotometer (AAS). As a result; 17 (51%) of the 33-bladder carcinoma tested patients revealed positive results for cytology, whereas using TRAP assay presence of the telomerase was positive in all of the tested bladder carcinoma cases. Only 30% (3 of 10) of the grade I tumors, 83.3% (5 of 6) of the grade II tumors and 50% (9 of 18) of the grade III tumors were diagnosed by cytology. The detection accuracy rates was statistically significant (100% for telomerase vs. 51% for cytology). The difference of relative telomerase activity (RTA) values between grades I, II and III was not statistically significant. Difference of serum zinc levels between the cases and control groups were significant (p= 0.04). Zinc levels in the both affected genders had dominant decreases, besides this shift was slightly more significant (p=0.04) in the female patients. In all, there was an inverse significant correlation between the RTA and serum zinc level in the case group (r= -0.060, p= 0.48). In conclusion our results showed a reverse relationship between zinc deficiency and increasing telomerase activity in our series of bladder cancer.

Key Words: Relative telomerase activity, zinc, bladder cancer.

INTRODUCTION

Bladder cancer is the fourth most common cancer among men and the ninth most common cancer among women. About 38.000 men and 13.000 women will develop the disease each year. The bladder cancer may occur at any age, but risk increases with advancing age, with 70% of male cancers and 75% of female cancers in individuals over the age of 65 years (1). Most bladder cancers are non-invasive or minimally invasive at the time of diagnosis. Women were found to be affected as advanced stage patients more than men. Urinary cytology plays an important role in detection and monitoring of bladder cancer. However, the aggregate, cytology methods pos-

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sess substantial limitations in detection of bladder cancer. It has considerable inter observer variability, depending on the adequate number of cells in the specimen. Low-grade cancers in which the cells often closely resemble normal bladder epithelial cells have a low detection rate by cytology (2). Telomeres, which represent the ends of eukaryotic cell chromosome arms, are specific DNA-protein complexes (3). Telomeric DNA usually consists of multiple repeating G-enriched nucleotide sequences that are directed towards the 3-end of the chromosome. In human chromosomes, telomeres are made up of hexanucleotide units, TTAGG (3) with the total length of 5-15 kbp (4). Telomeres seem to play a key role in stabilization of chromosomes upon replication by protecting their ends from exonucleases and ligases (5). In addition, telomeres prevent degradation and unwanted recombinations (6-8), such as fusion of the terminal regions of broken chromosomes. In normal somatic cells, telomeres undergo gradual shortening upon aging (9,10). After each replication cycle they lose up to 50 to 200 of nucleotides (11). Such shortening of chromosomes is due to the end-replication problem (12,13). Thus, DNA polymerase can synthesize the chain only in one direction from the 5'- to the 3'-end, but cannot initiate the synthesis at the very origin of the template. It can add nucleotides only to an already formed DNA region or to an RNA primer. After replication, RNA primers are removed and the short gap is filled with DNA polymerase. At the 5-end of a synthetic chain, this gap remains unfilled. As a result of which the newly synthesized DNA is shortened (14). Nevertheless, such shortening of chromosomes does not result in the loss of genetic information because this process is confined to the loss of senseless telomeric repeats alone (7). Gradual shortening of telomeres in normal somatic cells is mediated by a mechanism that counts off the replications and actually represents a mitotic clock (15). When telomeres reach a certain length, the cells begin to age and then die (16). In immortal cells, the shortening of telomeres does not take place. These cells display a practically unlimited capacity for replication, because they possess a mechanism, allowing to circumvent the problem of terminal replication. The length of telomeres in immortal cells is maintained at a certain level due to the addition of nucleotides to the 3-end by a special enzyme called telomerase (17). Telomerase is an enzyme responsible for maintaining the chromosome-end (18), which as commonly active in cancer. Telomerase is expressed in more than 90% of human cancers (19) whereas, in normal tissues or in benign tumors, telomerase is present in only a low percentage (20, 21). A polymerase chain reaction (PCR)-based on telomeric repeat amplification protocol (TRAP) assay on urine yielded a high sensitivity, but special collection techniques may be required (19).

The human catalytic core of telomerase is composed of an RNA component (human telomerase RNA, hTR), which serves as the template for telomere elongation and a protein subunit (human telomerase reverse transcriptase, hTERT) that acts to catalyze the telomere synthesis reaction. Therefore, telomerase is RNA-dependent DNA polymerase which uses its own RNA component as a template to elongate the 3' single-strand DNA extension of the chromosome terminus by the synthesis of telomeric repeats (22). One of the most interesting questions is whether a bipartite complex of hTERT and the template hTR performs the essential telomerase activity. Biochemical purification of the human and mouse telomerase suggested that mammalian telomerases were extracted as a complex having an approximate molecular weight of 1000 kD (Nakayama *et al.*, unpublished). Obviously, the predicted molecular weight of complex formed by hTRT (130 kD) and the human RNA template (9450 kD) is far less than the observed value if the telomerases were composed of one molecule of each component. One possibility is that the essential catalytic activity is performed by the hTERT/hRNA core complex (23), but telomerase-associated proteins, such as the TLP1/TP1 protein, associate with the core complex *in vivo* to modulate activity in response to various stimuli. The expression level of the hTERT genes is the primary determinant regulating the activity. Since most normal cells that lack detectable telomerase activity also lack hTERT expression, whereas hTR and some of the telomerase-associated proteins can be detected in these cells. These data have led to the hypothesis that hTERT expression is the essential factor of the telomerase activity and suggest that regulation of hTERT expression may be a key factor for the extension of the cellular replicative lifespan and cell immortalization (24, 25). However, given the multiple mechanisms by which telomerase are regulated, other mechanisms modulating telomerase activity might also contribute to determination which cells will be telomerase active. Thus telomerase-associated proteins and RNA template genes are candidates for

fine-tuning of telomerase activity. Telomerase is a target for diagnostic and therapeutic applications; while many studies have focused on how to depress telomerase activity (26, 27). However, how can inhibitors of telomerase be found? A classical approach to drug discovery is to test a battery of candidate small molecules for their effects on telomerase activity. It is also possible to identify the biological repressor of telomerase. Cell hybridization studies have shown that fusion of telomerase-expressing cell lacking telomerase activity results in hybrid cell that lack telomerase activity. Such dominant evidence proposed that somatic cell might express an inhibitor or repressor of telomerase activity (28). Zinc is an essential trace element and has a share in a number of cellular functions as a constituent of more than 200 enzymes and zinc-finger motifs in transcription factors (29). The role of zinc in proliferation of lymphocytes and other non-cancer cells has been approved (30). But other evidence shows that tumor size has a reverse relationship with zinc amount (31, 32). However, the effect of serum zinc level on telomerase activity has not been investigated. We examined relationship of zinc with the activity of telomerase in bladder cancer. We documented that enhancement of telomerase activity has a reverse relationship with amount of zinc.

MATERIALS AND METHODS

Patient selection and sampling

According to clinicopathological criteria such as haematuria and biochemical indexes, 33 bladder cancer diagnosed patients referred to Urmia Imam Hospital urology section, were selected. The voided urine and blood samples were gathered from selected patients. The blood was used for zinc concentration assay.

Voided urine specimens

30-50 ml of fresh urine specimens were spun at 2500 rpm for 5 mins (Beckman Company, AvantiTMJ-25). The supernatant was removed. The residual cells were washed in 1 ml phosphate-buffered saline (pH 7.4) at 4°C. They were then pelleted by centrifugation and were stored at -80°C until experimentation.

Extract preparation

Lysis reagent was added to the cell pellets until the pellet would become invisible indicating complete lyses. The samples were incubated on the ice for 30 mins. The lysate was then centrifuged at 16000 g for 20 mins at 4°C, and the supernatants were transferred to fresh tubes. 200 micro liters of extract were picked up from each sample in order to be used in protein concentration determination and subsequently telomerase assay, The remaining extract was immediately stored at -75°C.

Measurement of zinc

For preparation of zinc analysis, we selected 33 bladder cancer serum samples as case group and over 33 serum samples of healthy persons as the control group. 1 ml of patient's serum was diluted in 9 ml of distilled water and for standard preparation; 1 ml of the 90 mg/100 ml zinc standard solution was added to 99 ml distilled water in a volumetric flask. In the next step, different dilutions of standard solution were prepared. The zinc hollow cathode lamp was put in place, and adjusted to an operating currency of 10 mA. The most intense absorption line for zinc was at 213.9 nm. The absorbance of the standards and cases was measured, using the blank to set zero. A graph of absorbance was plotted applying nm versus ppm for calculating zinc concentration by using the standard calibrations. The concentration of zinc was reported as μg/dL.

Determination of protein concentration

Using distilled water, 10 dilutions of bovine serum albumin were prepared. 25-225 μL of protein were added to 775-975 μL distilled water. 800 μL of the prepared standard were supplied into a micro tube, followed by the addition of 200 μL Dye regent concentrate (BIO-RAD, Cat. #500-0002). After mixing and incubating for 5 mins at room temperature, OD was read in 595 nm. The extracted protein concentrations were determined using the BSA standard plot considering 595 nm absorbance versus the different concentrations of BSA.

Telomerase assay

Telomerase activity was determined using the TeloTAGGG Telomerase PCR ELISA^{plus} detection kit. (Roch, Cat. #2013789). The methodology is based on the modification of an original method described by Kim *et al.* (19). The TeloTAGGG Telomerase PCR ELISA^{plus} kit is a very sensitive *in vitro* assay system that used 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'). 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 are immobilized via the biotin label to streptavidin-coated micro titer plate. Immobilized amplicons were then detected using the antibody against digoxigenin, which was conjugated to horseradish peroxidase (Anti-DIG-HRP) and the sensitive peroxidase substrate TMB. The

telomeric repeat amplification protocol (TRAP) assay is performed according to manufacturer's protocol, which can be described briefly as follows: Reaction mixture (Biotinylated telomerase substrate P1-TS, optimized anchor-primer P2, nucleotides, and Taque DNA polymerase) was ready-to-use. 1 μL of heat-inactivated or test extracts were added to each tube (final volume, 50 μL). Heat inactivation was achieved through incubating 10 μL of each extract at 85°C for 10 mins, 1 μL of positive control template, containing high concentration of telomerase was added to its specific tube. As a negative control, lyses reagent (1 μL) was added to negative control tube. All the tubes were placed in a thermocycler block and incubated at 25°C for 20 mins and then at 94°C for 5 mins. Thirty cycles of PCR were performed, each cycle at 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 90 seconds. The PCR products were spliced into 2 aliquots, subsequently 10 μL of denaturing reagent were added into each of the separate reaction tubes and incubated at room temperature for 10 mins. 100 μL hybridization buffer-T- were added to one vial and the hybridization buffer IS (100 μL) to another one and were gently mixed. According to the pipetting scheme, each mixture (100 μL) was transferred into its well. The wells were incubated at 37°C on a shaker (300 rpm) for 2 hrs. After 2 hrs, the hybridization buffer was removed and then each well was washed 3 times with 250 μL of washing buffer. Anti-DIG-HRP working solution (100 μL) was added to any of the wells and then incubated at room temperature for 30 mins on a plated-shaker (300 rpm). Subsequently, the solution was discarded and washed 5 times using the washing buffer and consequently removed. 100 μL of the TMB substrate solution were pre-warmed in order to reach the room temperature and then were added to each of the wells. They were incubated for color development at room temperature for 15 mins while the plate was rotating at 300 rpm. Without removing the reacting substrates, 100 μL of stop reagent were added into each of the wells in order to stop color development. The absorbance of samples was measured at 450 nm using an ELISA micro titer plate reader within 15 mins after addition of the stop reagent. Relative telomerase activity (RTA) within different samples in an experiment is calculated using the following formula:

$$RTA= (A_s-A_{so}) / A_{sis} / (A_{TS8}-A_{TS8,0}) / A_{TS8.,IS}$$

Statistical analysis

All analyses were performed using SPSS 11 software. The *t* test for paired data was used to test the significance between the means of two groups. Analysis of variance (ANOVA) was used for further analysis, and if the differences were significant, the Fisher PLSD test was used for post analysis. All data were given as mean values±SD. The Relative Telomerase Activity, zinc level and other clinicopathological characteristics were assessed using the nonparametric Mann-Whitney U-test and the Kruskal-Wallis test.

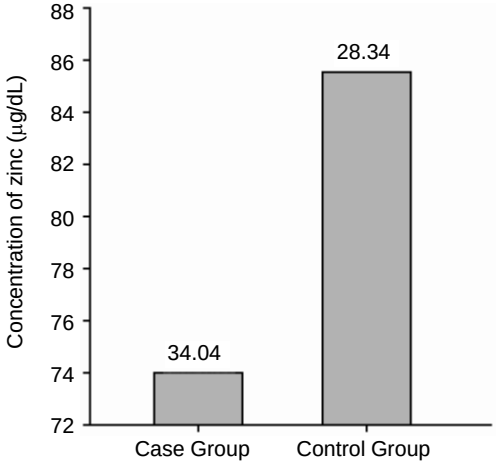
RESULTS

First we measured relative telomerase activity (RTA) in 33 consecutive bladder cancer patients. The RTA value in the case groups was 26.51±28.10 whereas there was not any telomerase activity detected in heated inactivated serum samples as control group. In 100% of patients, RTA was positive in voided urine samples with a high sensitivity (100%). The difference of RTA values within grade I, grade II and grade III cases was not statistically significant. Stage and grade were statistically associated with the RTA values in urine cells from patients (r=0.60, p=0.00; r=0.73, p=0.00; respectively). Correlation of grade with RTA value was stronger than stage (r=0.73 vs. r=0.60). Bladder cytology was positive in 17 of the 33 patients (51.1%) with cystoscopically diagnosed bladder tumor. In cytology method, the number of positive conformed cases regarding to grades I, II and III were 3 of 10, 5 of 6, 9 of 18, respectively. In low grade tumors, the number of cases diagnosed by cytopathology was low, whereas in cases where telomerase activity was high. Citological diagnosis was completely successful. There was a good diagnostic concordance between cytology and telomerase activity in voided urine cells of the patients. There was no significant correlation between tumor grades and telomerase activity whereas there was a strong correlation between RTA and stages (r=0.69, p=0.00).

Secondly, serum zinc concentration of bladder cancer patients and controls were measured and the obtained results for patients were 74.03±34.04 μg/dL and were 85.51±28.34 μg/dL for controls. The statistical analysis showed that there was a statistically significant difference between two groups (p=0.04) (Figure 1). In another analysis we could not find any association between tumor grades and zinc concentration in bladder cancer patients and also within the grades of tumor. Any statistically significant association between RTA and serum zinc levels was not observed. Regarding to the cancer stage results were also the same as the tumor grades. Regarding to age related variation, it was found that there is a reverse and statistically significant correlation between age and zinc concentration in bladder cancer patients (r=-0.30, p=0.04). On the other hand in the control group, there was a positive and significant correlation between serum zinc concentration and age (r=0.28, p=0.05). The serum zinc levels were also studied from the point of gender (Figure 2).

According to Mann-Whitney U test, serum zinc concentration of the cancerous males and non-cancerous males were 75.8 μg/dL and 85.5 μg/dL, respectively. However, the difference between two groups was not statistically significant (z=-1.3, p=0.19). In the cancerous females and non-cancerous females, serum zinc levels were 46.5 μg/dL and 78.5 μg/dL, respectively and this difference was meaningful and significant (z=-2.08 and p=0.03). The differed shift for the serum zinc levels regarding to female cancerous patients was more significant than that of for cancerous males (p=0.03 vs. p=0.19). Finally, between RTA and zinc levels reverse and significant correlation were found (r=-0.06, p=0.48), (Figure 3).

Figure 1: The serum zinc values were measured in case and control groups, using atomic absorption. The difference of serum zinc levels in two groups was statistically significant (p=0.04).



DISCUSSION

Telomerase compensates the loss of telomeric repeats, through synthesizing new chromosomal telomeres along with every cycle of DNA replication (4). Absence of telomerase activity and the resultant progressive shortening of the telomeres characterize the process of cellular senescence *in vivo* and *in vitro* (33). On the other hand, the acquiring of immortal phenotype for tumor cells is associated with the reactivation of telomerase expression, leading to telomere elongation, enabling unlimited cell division. It is estimated that approximately 66-97% of assayed human tumor samples have shown telomerase activity, including cancers of the lung (34), colon (35), stomach (36), liver (37), breast (38), prostate (39), brain (40), and endometrium (41). In the current study, we found that 100% of bladder cancer samples telomerase activity was detectable. This may indicate that telomerase assay may be more sensitive than cytology for fluid specimens containing only small numbers of malignant cells. The TRAP assay used in the current study can detect the enzyme activity in as few as 10 cancer cells. We therefore can say that this method has significantly increased the efficiency of diagnostic methods of bladder cancer (42).

It has been reported that the serum zinc level in 19 patients with prostate cancer was significantly lower than serum zinc level in 27 patients with prostatic hyperplasia (BPH) (43). According to another study (44) the zinc levels in patients with both cancer in the prostate and metastases were lower than in patients without metastases. Lower zinc levels were also observed in cancers other than prostate cancer. It is known that zinc deficiency in 47 head and neck cancer patients was associated with increased tumor size, overall stage of the cancer (45). Zinc levels were lower in lung cancer patients compared with advanced tumor than in those without advanced tumors (46). Also, in another study, two patients, one with larynx cancer and one with non-Hodgkin lymphoma, had 6 and 9 mg/dL zinc,

Figure 2: Comparison of serum zinc mean values in association with sex, determined in case and control groups, using atomic absorption. The difference between case and controls in females was statistically significant (r=-2.08, p=0.03).

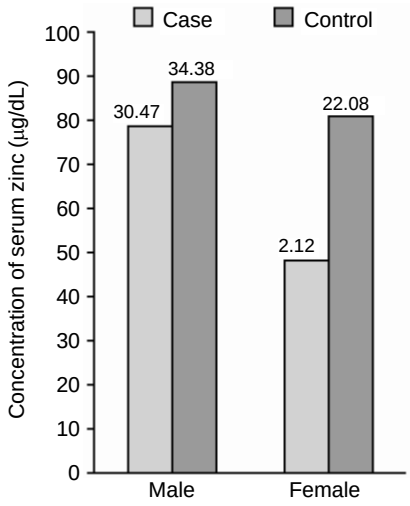
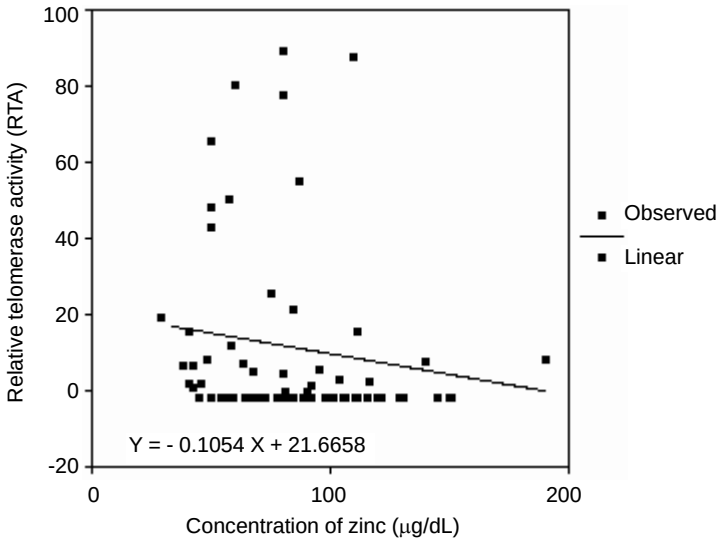


Figure 3: The correlation between RTA and serum zinc concentration in bladder cancer patients. The results show an inverse statistically significant relationship between two variables (r=-0.06, p=0.48).



although patients with newly diagnosed cancer had normal values of zinc (55). In the present study, difference of zinc between the bladder carcinoma group and control group was statistically significant (p=0.04) and it is worth nothing that in this group the reduction of zinc level in plasma was seen in the female patients was more than male patients. Some evidences indicate that zinc deficiency could be in favour of the patients. One such example is reported that survival of tumor-bearing mice was prolonged by administration of cadmium chloride, which induces zinc deficiency in the tumor cells (47). These results suggested that the restriction of zinc intake suppress tumor growth. However, Waalkes and Rehm, expert on tumors in rats, pointed out many years before, it was reported that oral cadmium treatment is clearly associated with tumours of the prostate while dietary zinc deficiency has complex, apparently inhibitory, effects on cadmium carcinogenesis (48, 49). Also, in “Yoshida Sarcoma” bearing rats, a significant inhibition of the tumor growth in size was observed by progesterone, which decrease zinc uptake (50). These paradoxical results with zinc are restricted to animals and test tube.

Some scientists believe that zinc deficiency may suppress tumor growth. In an experiment with two human prostate cancer cell lines it was shown that zinc could inhibit cell growth and increase apoptosis (51). Incubation of cell lines (LN Cap and PC-3) with physiological levels of zinc resulted in the marked inhibition of cell growth. These inhibitory effects of zinc have a satisfying correlation with the accumulation of zinc within the cells. The cell growth inhibition in the cells was accompanied by an increase in apoptosis. In another study, mice were fed *ad libitum* with either a zinc sufficient or a zinc deficient diet for 46 weeks, the tumor incidences for these groups were 57, and 100% respectively, in the esophagus, as compared to 17, 39, and 67% in zinc sufficient mice (52).

The Japanese researchers demonstrated that zinc induces an enhancement of telomerase activity in the human renal cell carcinoma (NRC-12) and prostatic cancer (DU145) cell lines. The maximum elevation of the activity was observed 6 hours after treatment with 100 mM solution of zinc; it was diminished by the addition of either metal chelators or cycloheximide. Other metals such as Cad and Cu also enhanced telomerase activity, but to a lesser extent (53). Because most normal cells that lack detectable telomerase activity also lack hTERT expression and hTR and some of the telomerase-associated proteins can be detected in these cells. These data have led to the hypothesis that hTERT expression is the essential factor of the telomerase activity and suggest that regulation of hTERT expression may be a key factor for the extension of the cellular replicative lifespan and cell immortalization (24,

25). Therefore, hTERT appears to be as a logical target for inhibition of telomerase. It is becoming increasingly clear that full transcriptional activity of the hTERT promoter requires the SP1 (contain zing-finger motif) transcription factor (54). Therefore, Japanese' results can explain up-regulation of telomerase activity by zinc. But the tumor suppressor protein p53 (contains zinc-finger protein) and the cell-cycle regulator E2F-1 negatively regulate hTERT transcription (55). Our results confirmed this matter. Telomerase is currently the best and most common general marker of cancer cells. Activity has been detected in every major category of human malignant neoplasia tested, exhibiting an overall prevalence of 85%. Accordingly, intense interest in the use of this marker for the purpose of diagnosis and/or prognosis has been generated in cancer research. This field is relatively young and future work is needed to improve and standardize current methods for detecting and quantitating telomerase activity. Also are needed more extensive clinical studies incorporating larger numbers of patients and longer periods of patient follow-up to help to clarify the potential diagnostic role of telomerase in the various types of human cancer. Elucidation of the mechanism by which zinc modulates the activity of telomerase might reveal not only a new aspect of the mechanism of regulation of telomerase activity in cancer cells, but also a new role of zinc in the regulation of the growth and senescence of cells. Further study on the mechanism of activation of telomerase by zinc as well as screening of other regulators of telomerase is required.

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