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Investigation of the Increased Copy Number of the Telomerase Catalytic Subunit Genes in Breast Cancer Paraffin-Embedded Samples in Ardabil

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ABSTRACT

Breast cancer is the most common cancer among the women and makes up one- third of cancers. Telomerase enzyme is a ribonucleoprotein with reverse transcriptase activity that adds TTAGGG repeated sequences to the ends of the chromosomes. The expression of the catalytic subunit of the enzyme (*hTERT*) in cancer cell lines and different human tumors is shown. So, in this study, the proliferation of copies of the *hTERT* gene in primary breast cancer was studied. Materials and Methods : Forty five Paraffin tissue samples from breast cancer patients and forty five Paraffin tissue samples from breast non-neoplastic patients was provided from Ardabil Imam Khomeini Hospital's Pathology section and Plasma Laboratory.DNA samples were extracted with phenol-chloroform manually, then Real Time PCR was done with *hTERT* Forward, and Reverse primers and *GAPDH* Forward, and Reverse. The tumor sample was compared with the non- tumor one to investigate proliferation of copies of the *hTERT* gene as a prognostic indicator in patients with primary breast cancer. Results: Real time PCR curve analysis for *hTERT* and *GAPDH* genes in tumor samples compared with non-tumor samples showed that, there is no statistical deference's between them (p=0.322). For data analysis Spss statistical software was used. Discussion: Our current studies on results of amplification *hTERT* gene, in comprising tumors samples with non-tumors is indication of early prognosis in primary breast cancer patients. While amplification of *hTERT* gene in breast cancer patients compared to the control group indicates that there is no statistical deference's between them and it couldn't be considered as prognosis factor for the primary breast cancer patients.

Key words: breast cancer, telomerase, *hTERT* gene.

INTRODUCTION

Breast cancer is the most common malignant disease in women all over the world and constitutes one-third of cancers and after lung cancer is considered the second leading cause of cancer mortality in women [1,2].

This disease is increased in Iran and since 1999 has the first country rank among recorded cancers [3,4]. Due to lack of organized screening and training programs for initial and early detection of breast cancer in Iran, about 70 % of Iranian women are diagnosed in dangerous stages of the disease [5]. According to the cancer registration, breast cancer in Ardabil is the most common one, following esophagus and stomach cancers [6].

Telomerase enzyme is a ribonucleoprotein complex of telomere-specific reverse transcriptase activity that uses an RNA template for adding TTAGGG repeated sequences to the ends of the chromosomes, and compensates for the loss of the telomere length. So telomeres are replicated by the telomerase enzyme [7,8,9,10,11,12,13].

Human telomerase enzyme activity is composed of the human telomerase reverse transcriptase (*hTERT*), human telomerase RNA (*hTR*) and Dyskryn [14].

Telomerase enzyme activity does not exist in normal somatic cells, but It is seen in 85 % - 90 % of human cancers, including more than 95 % of breast cancers, which is essential for continued proliferation [15]. A limited number of cancers (15%) preserve telomeres through recombination mechanism with an alternative of telomere length (ALT) [16].

hTERT is expressed only in the telomerase cells and is not expressed in differentiated cells [17,18]. *hTERT* gene will amplify abundantly in human tumors and tumor cell lines. This result implies that an increase in copy numbers of *hTERT* gene, can be involved in the regulation of telomerase expression in the immortalized cells [19,20]. It is also shown that *hTERT* is a decisive factor for controlling telomerase activity [18,21,22,23].

Reproduction of the catalytic subunit of the enzyme (*hTERT*) in cancer cell lines and different

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human tumors is shown. So, in this study, the proliferation of copies of the *hTERT* gene in primary breast cancer was studied.

Materials and Method

Forty five breast cancer formalin fixed paraffin embedded (FFPE) specimens obtained from the Department of surgery, Imam Hospital, Ardabil, Iran. All patients signed detailed consent forms before the study was conducted. The experimental samples were pre-made on standard slides with 5 micron thick FFPE tissues using the standard method by the Department of Pathology, Imam Hospital.

3-5 slices of 5 micron from selected samples placed in the 1.5 ml micro- tube and DNA samples were extracted with phenol-chloroform manually with using xylol, ethanol, Layzyz buffer, proteinase K, phenol saturated, chloroform - isoamyl alcohol, sodium acetate, isopropanol and the finally 50 micro- liter of distilled water added.

Then the sample for *hTERT* gene put through PCR, .For each process, 120 µl of buffer10 X, 36 µl of Mgcl2, 24 µl of dNTPs, 1 µl of primers Forward (F) and Reverse (R), 0.1 µl of the enzyme Taq DNA polymerase and 2 µl of DNA was used.

Sequences of primers used for F = 5´AGTGGAGACAGGCGCAT3´and R =5´ ATGGTGAGTGCTACATGGTGA3´.

Samples that were put through PCR processed in the beginning at 95˚C for 30 min, then 35 cycles of 95˚C for 30 sec, 35 cycles of 56˚ C for 30 sec, 35 cycles of 72˚C for 30 sec, and finally at 72˚C for 7 min. PCR products were electrophoresed at 0.01 agarose gel.

Then all of the samples in addition to *hTERT* gene for GAPDH gene with primer sequences F = 5´CTCTCTGCTCCTCCTGTTCGAC 3´ and R =5´TGAGCGATGTGGCTCGGCT 3´ were gone through Real Time PCR process. Forty five tumor samples and forty five non-tumor samples were compared to increased amplification of *hTERT* gene are reviewed as a prognostic marker for the Primary breast cancer patients.

Samples were put through Real Time PCR in the beginning at 50˚C for 2 min , then at 95˚C for 10 min, 60 cycles of 95˚C for 15 sec and finally 60 cycles of 60˚C 1 min.

Results:

In this experiment, Forty five paraffin-embedded tumor samples from breast cancer patients and 45 paraffin tissue samples from control group was provided and investigations were carried out on them, the results are as follows.

After the DNA tumor and non- tumor samples was extracted, PCR was performed to ensure the absence of primer dimmer. Then the PCR products were analyzed by gel electrophoresis.

To check the purity of the DNA extracted from nano- drop device was used. Absorbance reading DNA, extracted DNA quality can be achieved. Relative absorbance (Optical Density: OD) DNA at wavelengths 260 to 280 nm was measured. Higher OD at wavelengths 280/260 of 1.8 is an indicator of DNA purity. Different concentrations of DNA samples, using the formula C1.V1 = C2.V2 Nanodrop apparatus were identical and were given 20 ng micro liter [5].

Quantification increased amplification of *hTERT and GAPDH* gene in breast cancer patients compared with those without tumor was performed. Real time PCR curves amplification of *hTERT* gene in tumor and non- tumor samples, is shown in Figure 1.

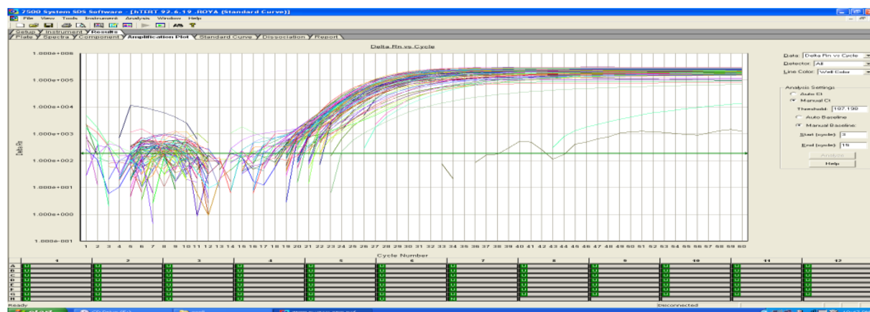

Fig. 1: The curve increased amplification of hTERT gene in samples from patients and control group in the experimental Real time PCR. The amount of fluorescence emitted increased during the cycle of the of increased PCR products amounts.

The average numbers of hTERT on group of Cancer Patient are 19 ± 4.8 and in control group these numbers were 18.6 ± 4 which show that, there is no visible deference in computed average numbers between control group with] group of cancer patients (Figure 2). In cancer patients and control groups the OR=1.4 and the confidence interval (CT) is equal with 0.9 – 2.2 which shows no noticeable deference between two groups.

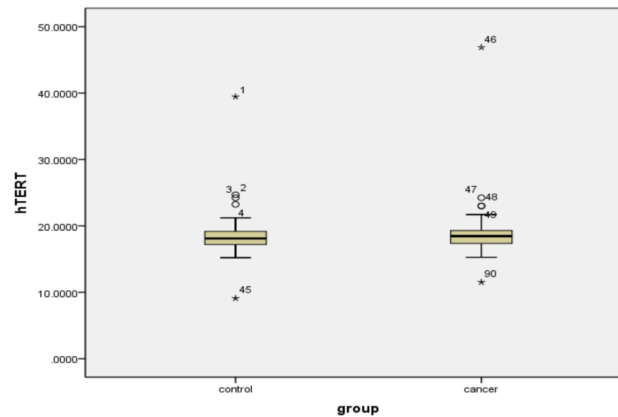

Fig. 2: Amplification of hTERT gene in breast cancer patients compared to the Control group indicates that, there is no statistical association existed between them.

Descriptive data of all samples of the Cancer Patients, at stage one, 2 cases (% 4.4), at stage two, 25 cases (% 55.6) at stage three, 18 cases (% 40) are shown (Table1).

Table 1: The condition of the stages.

		Frequency	Percent
Valid	1	2	4.4
	2	25	55.6
	3	18	40.0
	Total	45	100.0

Interpretation of results was done using the comparative CT method for quantitation according to this arithmetic formulas:

$\Delta CT = C_{T\ target} - C_{T\ refrence}$

$hTERT_{\ adjusted} = 2^{-\Delta ct}$

The mean total in Cancer Groups 20273 ± 135993 and in control group it is 91 ± 601 which shown that there is no deference between two groups (p=0.322).

Discussion:

Breast cancer is the most common cancer in women the world [24]. Telomerase is a ribonucleoprotein enzyme consisting of two parts. The first part, subunit active catalytic protein, *hTERT* reverse transcriptase activity that adds telomere repeated sequences to the ends of the chromosomes and that is essential to the continued growth of tumor cells. *hTERT* gene is increasingly amplified in human tumors and tumor cell lines.

The secondary part of the template RNA in humans is called *hTR* and used as a template for telomere synthesis. Telomerase is an therapy target for ideal anticancer because of its activities in more than 90 % of human cancers, there are including more than 95 % breast cancer, whereas most somatic cells are indistinguishable [25].

Thomas and his colleagues, have also concluded the amplification of *hTERT* gene in Lanfobelastic acute leukemia (ALL) and non Lanfobelastic leukemia (ANLL). Quantitative analysis showed that leukemia cells have many numbers of the copies of *hTERT*, *hTERC* are normal PBL. The results indicate that telomerase activity in leukemia cells is associated with amplification of *hTERT* gene, *hTERC* [26].

Richard and his colleagues in 2005 to increase the hTERT gene copy number in samples from 64 colorectal carcinomas were reviewed and increased copy number (>= 3 hTERT gene copies/ nucleus) were observed in 31(48%) cases. No correlation was found between hTERT gene copy number and hTERT RNA expression or telomerase activity. Data show increase in copy number of the hTERT gene in colorectal carcinoma was the result of unstable telomerase activity levels was not associated [27].

Ying and colleagues studies, amplification of *hTERT* gene was observed in cell lines tumors and various human cancers, as well as a mechanism of telomerase activation is introduced. It is remarkable that telomerase activity increases in both of the primary cells and cancer [28].

In 2008, based on studies in Russia, the hTERT DNA copy number of the 33 studied tumors compared to normal tissues was unchanged. Similar results was achieved with squamous cell cervical carcinomas (SCC) cell lines in human papillom virus(HPV)genomes.However, the activation of hTERT expression was discovered in 80% of cases (37/46, p<0.001). There was no relationship between the degree of mRNA increase and the tumor size and/or prevalence metastases. No hTERT gene expression was shown in 20% of cases(9/46), while the control GAPDH expression has remained unchanged. The conclusion was that, the frequent activation of hTERT expression in SCC is not linked with gene amplification [29].

Zhang and colleagues Saretzki have been reported a link between two *TERT* genes and telomerase activity in multiple cell line and primary tumors, the study Palmqvist primary tumors of samples and other studies of the stability of human breast epithelial cells and human foreskin fibroblasts no correlation were not observed between the copies number of *hTERT* genes and telomerase activity. No relationship could be observed a complex the telomerase enzyme, consisting of components and multiple evidence, telomerase activity is limited to the level two components (*hTERT* and *hTR*) [30].

Top of Form:

In a variety of tumors, increasing the copy number of the *TERT* has clinical relevance and prognosis For example, Zhu and his colleagues have shown that Lung Cancer Patients with increased amplification of *hTERT* with reoccurs will not last long. In melanoma, copies of increased amplification of *hTERT* correlated to subunits of melanoma and era of metastasis. Also the research shows that, Amplefication of *hTRET* in different cell line normally is independent of telomerase activities [31].

Our current studies on results of amplification *hTERT* gene, in comprising tumors samples with non-tumors is indication of early prognosis in primary breast cancer patients. While amplification of *hTERT* gene in breast cancer patients compared to the control group indicates that there is no statistical deference's between them and it couldn't be considered as prognosis factor for the primary breast cancer patients.

According to conducted research it could be suggested that the *hTERT* gene may be promoted through telomerase activity, but increasing the copy number in various tumor cell line is not always dependent on telomerase activity and it likely could have an independent activity.

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