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The relationship between tissue differentiation-inducing non-protein coding RNA expression and tumor size in patients with breast cancer

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ABSTRACT

Cancer is one of the leading causes of human death worldwide. One of the most common types of malignancy among women is breast cancer, which is the third most common cancer in the world after lung and stomach cancer. This study aimed to evaluate the expression level of Tissue Differentiation-Inducing Non-Protein Coding RNA (*TINCR*) in adjacent tumor and non-tumor tissues of 60 women with invasive ductal breast cancer. The relationship between *TINCR* expression and the clinical characteristics of patients has also been studied. For this purpose, total RNA was isolated from breast cancer patients' adjacent tumor and non-tumor tissue. RT Prime Script reagent was then used to convert total RNA to cDNA. The qRT-PCR quantified the *TINCR* expression level and analyzed the results by paired t-test. In addition, ROC curve analysis was used to evaluate the biomarker power of *TINCR* in breast cancer tumor tissues. According to the results, a decrease in the level of *TINCR* was obtained in the tumor tissue of breast cancer patients compared to the adjacent non-tumor tissue ($P<0.001$). *TINCR* expression was negatively correlated with tumor size and lymph node metastasis in breast cancer tumor tissue. In general, the decrease in the expression level of *TINCR* in the tumor tissue of breast cancer patients shows that its expression level can differentiate the adjacent tumor and non-tumor tissue from each other. In addition, *TINCR* has a lower expression level in breast cancer patients with large tumors, lymph node metastasis, and luminal subgroups A and B.

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Introduction

Cancer is one of the leading causes of human death worldwide. One of the most common types of malignancy among women is breast cancer, which is the third most common cancer in the world after lung and stomach cancer (1). The study of cancer prevalence and mortality in countries increases health system costs, especially in low- and middle-income countries (2). In this way, with the aging of the population and the adoption of the Western lifestyle, breast cancer will become an important economic challenge in these countries (3). Five to ten percent of breast cancers are due to predisposing mutations in reproductive cells, and more than 50 percent of these cases occur in BRCA1 and BRCA2 genes. Age Standardized Rate (ASR) in China is 0.82 per 100,000 for men and 24 per 100,000 for women. Many people die of breast cancer every year in different countries, including China (1). If this disease is diagnosed on time, its control and treatment will be faster, and its success will be more. Various studies have been conducted to identify oncogenic and tumor suppressor factors in cancers, especially breast cancer, which can help identify better treatment methods (4).

Long non-coding RNAs (long non-coding RNAs or lncRNAs) can be mentioned among these factors. lncRNAs are a group of transcripts with a length of more than 200 nucleotides, which are considered important regulators of gene expression and carcinogenesis (5). These molecules participate in various cellular processes, such as epigenetic regulation of gene expression and modulation of expression at the level of transcription and post-transcription (6). This way, lncRNAs can affect cell proliferation, apoptosis, migration, and genome stability. These molecules usually play the role of oncogenes or tumor suppressors in tumorigenesis. Recently, some lncRNAs have been identified whose role in carcinogenesis depends on the tissue where they are expressed. Among these lncRNAs, we can mention terminal differentiation-inducing non-coding RNA (*TINCR*) (7). The *TINCR* gene is 3,700 nucleotides long, and its expression occurs in the final stage of human epidermis differentiation. The expression of this ANRcnl led to the regulation of the expression of essential genes involved in the differentiation process (8).

Various studies have shown that *TINCR* has specific expression in the skin, placenta, and esophagus (8, 9). Based on recent studies, *TINCR* in tumors of different cancers is expressed differently, which shows the importance of studying this molecule in cancer and its role as a new biomarker in diagnosing and treating this disease (9). In this way, the present study aims to investigate and compare the expression level of *TINCR* in tumor tissue and adjacent non-tumor tissue in women with breast cancer. Also, the relationship between this gene's expression and the disease's clinical characteristics and the determination of the *TINCR* biomarker in tumor tissue are investigated.

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Materials and Methods

Sampling

In this study, 60 women with non-invasive ductal carcinoma of the breast were referred, and samples of tumor tissue and adjacent non-tumor tissue with a distance of 6-10 cm from the tumor tissue were obtained by random sampling. The sample size was calculated using G Power software, and 53 pairs of adjacent tumor and non-tumor tissue samples were estimated with a 95% confidence factor, 90% power, and 0.5 effect size. In this study, sampling was done from 60 patients, taking into account a 12% possible drop in samples.

Total RNA extraction and reverse transcription

From all pairs of tissue samples and patients, RNA was extracted using the QIAGEN kit (USA) and then kept at -80°C until the next steps. A spectrophotometer (Eppendorf, Germany) and agarose gel electrophoresis were used for quantitative and qualitative evaluation of the extracted RNA, respectively. Reverse transcription was also performed using the Prime Script kit (China) to synthesize DNA from the extracted RNA.

The qPCR reaction

TINCR and GAPDH primers were designed as internal control genes using Gene Runner software and the NCBI website, and the sequence of these primers is listed in Table 1. The qPCR reaction was performed using the SYBR Green method using the AMPLIQON master mix (Denmark) with the Step One PlusTM device (America) on the obtained cDNA.

Statistical analysis

The results obtained from PCR were analyzed with the help of SPSS Graph, Pad Prism, and MedCale software, and G Power software was also used to calculate the sample size.

In addition, the paired t-test was used to compare the average gene expression in adjacent tumor and non-tumor tissues. To compare the expression level of *TINCR* in the tumor tissue compared to the adjacent non-tumor tissue, the delta CT method (Fold change calculation) was used. To check the biomarker power of *TINCR*, ROC curve analysis was used in the tumor tissue of patients, and each sample was analyzed in duplicate for each pair of primers. In these data, a P-value less than 0.05 is defined as significant.

Results

This study was conducted on 50 women with non-invasive ductal carcinoma of the breast, whose age at diagnosis was between 26 and 78 years. Examining the expression level of *TINCR* in the tumor and adjacent tumor tissue samples of patients showed that this gene's expression level in tumor tissue was significantly reduced compared to the adjacent non-tumor tissue ($p<0.001$). As shown in Figure 1, the reduction ratio of *TINCR* expression in the tumor was five times that of adjacent non-tumor tissue.

The relationship between *TINCR* expression and the clinicopathological characteristics of patients is listed in Table 2. According to these data, INCR expression level was significantly associated with tumor size (P=0.005) and metastasis in lymph nodes (P=0.003). Based on Table 2, no significant relationship was observed between other clinical characteristics of patients and the expression level of this gene. In addition, ROC curve analysis was compared to check the biomarker power of *TINCR* in the tumor tissue of patients (Figure 2); the area under the curve was 0.93, sensitivity was 97.3%, and specificity 98% ($p<0.001$).

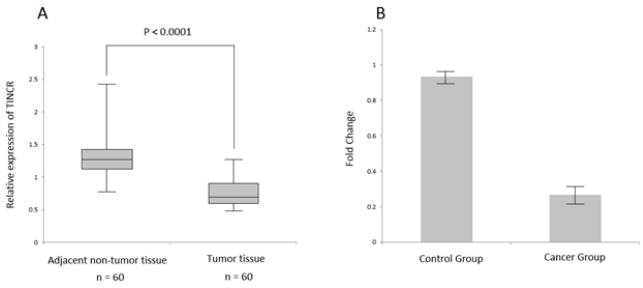

Figure 1. *TINCR* expression level in women with breast cancer; (A) *TINCR* expression level in tumor tissue of breast cancer patients in comparison with non-tumor tissue, (B) Fold change diagram that shows the expression level of *TINCR* in the tumor tissue of breast cancer patients compared to the normal state (each sample was tested in duplicate for each pair of primers).

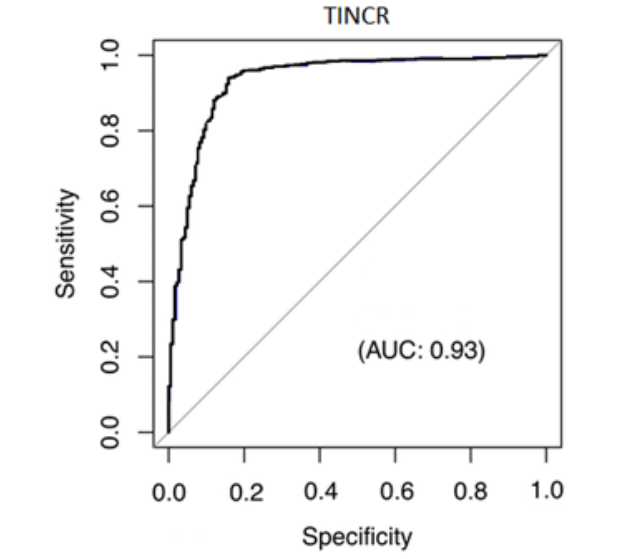

Figure 2. ROC curve in patient tissue samples (AUC: area under the curve).

Table 1. Primer sequences, product length, and annealing temperature for cTnI gene and reference gene.

Gene	Primer Sequence (5'-3')	Product length	Annealing temp.
TINCR	F: GCCCAAATGACAAGGGCTCTTT	114 bp	58°C
	R: AGTCGCTCRRCTCCAGGGCC		
GAPDH	F: CATGCTGCAGTCTGATTGCG	218 bp	60°C
	R: CATGAATGRAGGTCCCCGGC		

Table 2. Relationship between *TINCR* gene expression and clinical characteristics of breast cancer patients.

Pathological Characteristics	Number	Mean of *TINCR* gene expression	*P-value*
Tumor Size (cm)			
<2	11	0.341	
2-5	53	0.052	0.005*
>5	6	0.014	
Histological Degree			
Well differentiated	16	0.152	
Moderately distinguished	51	0.241	0.162
Poorly differentiated	10	0.076	
Molecular Sub-type			
Luminal A	46	0.319	
Luminal B	22	0.255	
HER2	5	0.21	0.651
Triple-negative Breast Cancer	3	0.22	
Unknown	11	0.442	
Identification and Disease Stage			
1	33	0.219	
2	17	0.178	
3	20	0.202	0.411
4	23	0.331	
Unknown	2	0.189	
Involvement of Lymph Nodes			
Positive	45	0.199	0.344
Negative	36	0.178	
Presence of Metastasis in Lymph Nodes			
Positive	17	0.031	0.003*
Negative	61	0.412	
Peripheral Invasion			
Positive	53	0.241	0.221
Negative	25	0.206	
Invasion of Lymphatic Vessels			
Positive	16	0.216	0.606
Negative	66	0.156	

Discussion

Studies conducted in recent years have shown that *TINCR* is an important regulatory molecule for epithelial differentiation (10, 11). By forming the *TINCR*-STAU1 complex, this molecule regulates the genes involved in the differentiation process, such as the KRT80 gene (12). The obtained evidence shows that *TINCR* is engaged in developing malignancy, tumor growth, metastasis, and resistance to chemotherapy and radiotherapy. Several studies have been conducted to evaluate the expression of this gene in cancer cell lines (12-14). These studies assessed the effects of silencing or overexpression of this lncRNA in cell proliferation, apoptosis, and invasive properties of these cells (13). In addition, the studies conducted on the mechanism of action of *TINCR* in tumorigenesis show that depending on the type of cancer, the gene's expression change can play a role in the process of tumorigenesis by interfering with different signaling pathways (15). For example, *TINCR* in colorectal cancer leads to an increase in the proliferation of cancer cells through the interaction with the STAU1 protein and the effect of increasing the stability and expression of the CDKN2B gene transcript (16).

Increased expression of *TINCR* has been observed in some malignancies, such as gastric cancer (17), hepatocellular carcinoma (18, 19), and esophageal squamous cell carcinoma (20). Studies conducted on some other cancers (21-29) have shown conflicting results. For example, in colorectal cancer (16), lung cancer (30, 31), and breast cancer (32) in some studies, a decrease has been observed, and in others, an increase in lncRNA expression has been observed in tumor samples or cancer cell lines. Contrary to this, studies of several other reports have shown that decreased expression levels of *TINCR* can play an essential role in the metastasis and progression of prostate cancer, oral squamous cell carcinoma, and squamous cell carcinoma of the skin. According to these reports, in the present study, a 5-fold decrease in the expression level of *TINCR* was found in breast cancer tumors compared to the adjacent non-tumor tissue. Some recent findings have proved that *TINCR* shows specific expression in breast cancer molecular subtypes. Specifically, in the *HER-2* positive subtype, there was an increase in expression (14). In the

present study, 90% of the participating patients were in luminal subgroups A and B; while 6% of patients were *HER-2*-positive and 4% were triple-negative, which confirms the specific expression of *TINCR* in *HER-2*-positive molecular subtypes in breast cancer. Also, a low level of *TINCR* expression has been observed in patients with large tumors and involvement of lymph node metastasis which shows the protective role of *TINCR* in the progression of breast cancer. In addition, the ROC curve analysis in the tumor tissue of patients also showed that the decrease in the expression level of *TINCR* could distinguish tumor tissue from non-tumor tissue of breast cancer.

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