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Plasma Circulating Terminal Differentiation-Induced Non-Coding RNA Serves as a Biomarker in Breast Cancer

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ABSTRACT
Background: Breast cancer is identified as the most common malignancy and cause of cancer related death worldwide. This study aimed to evaluate the expression level and diagnostic power of lncRNA plasma *TINCR* in breast cancer patients compared with healthy controls.
Materials and Methods: Fifty-eight women diagnosed with invasive ductal carcinoma and fifty healthy age matched controls were included in the study. TRIzol® LS regent was used to isolate the total RNA from whole plasma. Total RNA was converted to cDNA using Prime Script™ RT reagent kit and the expression levels of *TINCR* were quantified by qRT-PCR.
Results: Low levels of *TINCR* lncRNA were observed in plasma of breast cancer patients compared with control subjects. Plasma *TINCR* level was also positively correlated with diagnostic age of breast cancer patients.
Conclusion: Low level of plasma *TINCR* could discriminate breast cancer patients from healthy control subjects.

Keywords: Breast cancer; *TINCR*; Plasma

INTRODUCTION

Breast cancer (BC) is known as the most prevalent malignancy and cause of cancer related death worldwide, affecting over one million women every year. The average incidence of primary breast cancer of Iranian females in the year 2015 was 22.6 (95%CI 22.1–23.1) per 100,000 females, with an age-standardized rate (ASR) of 27.4 (95%CI 22.5–35.9)[1] 1 Compared with developed countries, high rates of breast cancer patients in developing Asian countries, such as Iran, are young (10% for developed vs. 25% for developing countries, respectively) [2-4].The breast cancer incidence increases considerably with age and its peak occurs in age of menopause [5]. In other hands, after gender, age is the most important known risk factor for breast cancer [6].

Long non-coding RNAs (lncRNAs) comprise a group of transcripts, generally longer than 200 bp, which could modulate chromatin structure and regulate gene expression at both transcriptional and posttranscriptional levels[7]. Dysregulation of lncRNAs can be associated with several kinds of human malignancies[8] which this potential of lncRNAs could serve as promising biomarker for cancer detection. Terminal differentiation–induced non-coding RNA (*TINCR*) regulates the differentiation of human keratinocytes by stabilizing a subset of mRNAs required for terminal epidermal differentiation[9].

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TINCR is usually kept at extremely low levels in epidermal stem cells, but considerably increases upon differentiation[9]. According to the reports by Human Protein Atlas RNA-seq dataset, TINCR has specific expression in placenta, esophagus and skin[10]. TINCR affects multiple cellular processes including proliferation, growth and apoptosis, and was recently linked to the pathogenesis of cancers. In gastric cancer cell lines, the nuclear transcription factor SP1 has a prominent role in induction of TINCR expression which is mediated through its interaction with STAU1 protein [11]. TINCR decreases expression of miR-21 in non-small cell lung cancer (NSCLC) and could suppress invasion and migration [12]. Moreover, a number of studies have showed the diagnostic power of TINCR in different cancer types which the best results have been reported in colorectal cancer[13]. Furthermore, expression levels of this lncRNA also could distinguish oral squamous cell carcinoma tissues from adjacent non-cancerous tissues [14].

Understanding the molecular basis and function of lncRNAs may help in early detection and can provide new insights into the therapy of breast cancer. Therefore, development of blood based and invasive markers is critical for early detection of breast cancer. Thus, we aimed to evaluate the expression level and biomarker power of circulating *TINCR* lncRNA in plasma of breast cancer patients compared with control subjects.

MATERIALS AND METHODS

Subjects

In total fifty-eight women who were newly diagnosed with invasive ductal carcinoma at Noor-e Nejat Hospital (Tabriz, Iran) from November 2016 to October 2019, were enrolled in this study. Plasma samples from 58 BC patients, before surgery as well as 50 healthy age matched controls were collected in sodium heparin tubes. Thereafter, plasma was obtained by centrifugation at 1300 × *g* at 4°C for 10 min and stored at -80°C. None of the participants received chemotherapy or radiotherapy before surgery and sampling. Current study was approved by the Ethics Committee of Tabriz University (ID: IR.TABRIZU.REC.1398.016) and written informed consent was obtained from all participants.

Reverse transcription and quantitative real-time PCR

Total RNA was extracted from 250 µL plasma using TRIzol LS (Invitrogen, Germany) reagent according to the manufacturer’s instructions. Three µL (50 pmol/l) of *Caenorhabditis elegans* miR-39 (cel-miR-39) (Norgen Biotek, Canada) at a final concentration of 10^{-4} pmol/µl was spiked into each plasma sample during the extraction step. This exogenous synthetic miRNA has been used for monitoring the efficiency of RNA extraction and sample quality. Picodrop microliter spectrophotometer (OEM, Hinxton, UK) and agarose gel electrophoresis were used for evaluation of quantity and quality of extracted RNA. Thereafter, cDNAs were synthesized by Prime Script™ RT reagent kit (Takara Bio, Shiga, Japan) from 8 µL total RNA according to the manufacturer's protocol, with one minor modification during the initial PolyA tailing step, in total reaction volum of 20 µL. Reverse transcription reaction mixture was incubated at 25°C for 10 minutes, at 42°C for 50 minutes, and at 85°C for 5 minutes and then was held at 4°C. The expression levels of *TINCR* were analyzed by RT-qPCR using the SYBR-Green method by Master Mix Green (RealQ plus 2x, AMPLIQON) in a Step One Plus™ Real-Time PCR System (Applied Biosystems) in all samples. In qPCR assay of 20 µL total reaction, 2 µL of a 5-fold dilution of RT product was used as a template. The template was mixed with 10 µL 2x Master Mix Green (RealQ plus 2x, AMPLIQON), 0.25 M universal reverse primer (only for cel-miR-39 reactions) and 0.2 M gene-specific primers. For normalization of *TINCR* lncRNA levels, *U6* gene was used in plasma. The expression level and fold change value of TINCR lncRNA were calculated using the $2^{-\Delta Ct}$ (15) and $2^{-\Delta\Delta Ct}$ methods (16), respectively, where $\Delta Ct = (Ct_{target} - Ct_{reference})$ and $\Delta\Delta Ct = \Delta Ct_{patients} - \Delta Ct_{control\ subjects}$. The PCR conditions consisted of an initial 5 min denaturation at 95°C, followed by 35 cycles of 95°C for 20 sec, 60°C for 30 sec, 72°C for 20 sec, and then a final extension of 5 min. Primer sequences were as follows:

TINCR: (5´-CACACTGACTCTTCCTGCTC-3´ and 5´-CAAACAAAGAAGGTGGGACAT-3´)

U6: (5´-CTCGCTTCGGCAGCACAT-3´ and 5´-GGAACGCTTCACGAATTTGC-3´)

Statistical analysis

Statistical analyses were conducted using SPSS 16.0 and Graph Pad Prism 8.0 software. For comparison between groups, T-test was applied. Receiver operating characteristic (ROC) and the area under the curve was used to determine the biomarker potency and optimal values of differential expression of *TINCR* in plasma samples. Data are presented as mean± standard deviation. P≤0.05 was considered to indicate a statistically significant difference.

RESULTS

Association of the TINCR expression levels with clinicopathological data

The association of TINCR expression level with the clinicopathological characteristics of the patients was presented in Table 1. Our results showed no significant association of TINCR expression level with these tumor clinicopathological features.

***TINCR* was deregulated in plasma of breast cancer patients**

In current study, the plasma level of *TINCR* was evaluated in 58 females diagnosed with invasive ductal carcinoma and 50 healthy age-matched control participants. The mean diagnostic age of the included 58 BC cases was 47.41 years of old and also was 48.25 for control subjects (p>0.05). The range of age at diagnosis in BC cases was between 26 and 78 years old. Compared to control subjects, notable downregulation of *TINCR* was observed in plasma of breast cancer patients (p<0.0001) (Figure 1, a). ROC curve analysis was performed to evaluate the diagnostic potential of *TINCR* in plasma of breast cancer patients (Figure 1, b). The diagnostic power between patients and controls was depicted by the AUC. This value was 0.886 (95% CI 0.816–0.956) with 90% specificity, 81% sensitivity (p<0.001) and ≤0.26 cut-off point value.

Correlation of the plasma TINCR level with age at diagnosis

The Pearson's correlation coefficient was used to reveal possible correlation of plasma TINCR level with age at diagnosis. It showed a positive correlation between plasma TINCR and diagnostic age of breast cancer patients (r=0.5169, p<0.0001) (Figure 2), indicating that aging drives elevation of plasma TINCR level. However, no correlation was observed between the age of healthy control individuals and TINCR plasma level.

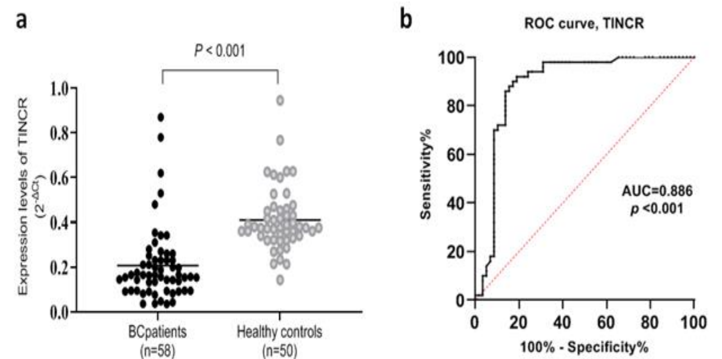

Figure 1. Plasma level and diagnostic potential of *TINCR* in breast cancer patients. (a) Dot histogram normalized with U6 relative expression level and (b) ROC curve of *TINCR* in plasma of breast cancer patients compared with control subjects, AUC: area under the curve.

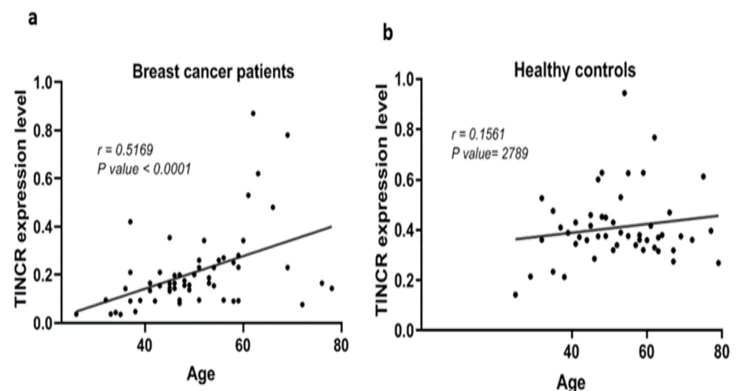

Figure 2. Correlation analysis of TINCR expression level and age in breast cancer. TINCR expression level was positively correlated with age of breast cancer patients (a), but had no correlation with age of healthy control group (b), r: Pearson's correlation coefficient.

Table 1. The association of TINCR expression levels with clinicopathological features of BC patients

Disease features	N	TINCR (Mean)	P
Tumor size			
<2cm	6	0.298	0.08
2-5cm	49	0.192	
>5cm	3	0.115	
Histological grade			
Well differentiated	8	0.251	
Moderately differentiated	46	0.288	0.54
Poorly differentiated	4	0.103	
Molecular subtype			
Luminal A	41	0.213	
Luminal B	14	0.275	0.721
HER2	1	0.198	
TNBC	0	0	
Unknown	2	0.164	
Clinical Stage at diagnosis			
I	22	0.219	
II	10	0.243	
III	12	0.193	0.362
IV	13	0.365	
Unknown	1	0.139	
Lymph nodes involvement			
Positive	33	0.341	0.186
Negative	25	0.105	
Lymph nodes metastasis			
Positive	18	0.102	0.078
Negative	40	0.374	
Lymphovascular invasion			
Positive	45	0.214	0.548
Negative	13	0.271	

DISCUSSION

Breast cancer is the main cause of cancer deaths in Asians, 15% of all cancer deaths in women, when compared with the rest of the world (17). Notably, the breast cancer prevalence in females under 40 years of age is much higher, indicating that the breast cancer incidence has a trend toward younger age (3). Additionally, the diagnostic age of breast cancer patients in developing countries is significantly less than those in developed nations (2). Breast cancer arising at a younger age might be more aggressive due to biological differences and more severe disease course. For instance, young patients mainly have a higher rate of Ipsilateral breast tumor recurrence which is associated with subsequent distant metastasis in this age group (18, 19). Moreover, the risk of mortality has been increased 5% for every 1-year reduction in age for younger patients compared with who aged 35 to 50 years (20). It is noteworthy that the increased delay in breast cancer diagnosis of young women is associated with more advanced stage disease and a poor survival rate (21). For this reason, most young breast cancer patients who diagnosed with early stage disease would survive many years with subsequent treatments. Twenty two percent (13 in 58) of our breast cancer cases were under 40 years of age which is considerably high when compared with Western counterparts (22). Due to the higher rate of dense breasts in young women, mammograms have a significantly lower sensitivity for breast cancer (23). Moreover, these screening methods mainly have harmful effects which are not recommended for young women (24). Therefore, novel blood-based and invasive markers are required for improve the early detection of breast cancer.

These biomarkers could present the potential for early detection of diseases, with a break in disease progression.

In current study, TINCR was detectable in plasma of all patients and healthy controls. Our data showed the significant low level of *TINCR* in plasma of breast cancer cases compared with healthy controls. The aberrant expression of *TINCR* has been observed in various human cancers. For instance, TINCR was upregulated in colorectal (25) and gastric (26) cancers. However, downregulation of *TINCR* has played an important role in invasion and metastasis in a number of cancers, such as oral (27), lung (28) and prostate (29) cancers. Wang and coauthors revealed that the plasma *TINCR* was upregulated in triple negative breast cancer (TNBC) patients (30).

However, the study by Xu identified *TINCR* as a subtype specific lncRNA associated with HER-2 positive subtype of breast cancer (31). It is noteworthy that 95% of the BC patients in our study were characterized as luminal A and B subtypes; while 3.4% were HER-2 positive and 1.7% were TNBC. As a result, our study revealed the low plasma level of *TINCR* in luminal A and B subtypes of BC, suggesting the subtype specificity feature of *TINCR* in breast cancer. However, *TINCR* expression status in plasma of luminal subtype breast cancer and its underlying mechanism is still unclear. Moreover, our data revealed that the plasma *TINCR* level had positive correlation with diagnostic age of patients. In other hands, young breast cancer patients have lower plasma level of *TINCR* than older participants. Our data also highlighted the diagnostic value of plasma *TINCR* which could distinguish BC patients from healthy controls.

CONCLUSION

In conclusion, our study showed the subtype specificity feature of *TINCR* in patients with invasive ductal carcinoma. Moreover, our findings suggested that the low plasma levels of *TINCR* can discriminate women with invasive ductal carcinoma from healthy individuals. Therefore, *TINCR* could be a blood based and invasive biomarker to improve early detection of breast cancer, especially for young women. Further research is needed to evaluate the expression analysis of TINCR in plasma of other types of breast cancer patients including cases who are candidate for therapy as well as patients after surgery.

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CONFLICT OF INTEREST

All the authors declare that there is not any conflict of interest.

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