Circulating CYTOR as a Potential Biomarker in Breast Cancer

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Long non-coding RNAs (lncRNAs) are newly introduced as tumor-related molecules. They are being considered as potential diagnostic and therapeutic targets in cancer studies. Here, we have assessed the importance of CYTOR (linc00152) as a biomarker for the detection of breast cancer in both tumoral tissue and plasma. The relative expression of breast cancer-associated CYTOR was measured in 20 tumoral and paired margin tissues, and moreover, in 80 plasma samples by real-time-polymerase chain reaction. In addition, plasma levels of CA 15-3 were measured using the ELISA test. The receiver operating characteristic curve (ROC) was applied for assessing the sensitivity and specificity of tested biomarkers. The results of the present study disclosed significantly increases in the CYTOR expression levels in tumor tissue with relative quantitation (RQ) equals to 5.15 (P<0.001) and in plasma (RQ =3.03, P<0.01) of breast cancer patients. The area under the ROC curve (AUC) of circulating CYTOR was 0.907 (95% CI: 0.842-0.972, P<0.001), while it was 0.868 (95% CI: 0.783–0.952, P<0.001) for CA 15-3. Based on these findings, we suggest lncRNA CYTOR as a new potential biomarker for breast cancer detection in both solid tumor tissue and plasma.

**Key words:** Breast cancer, lncRNA, CYTOR, biomarker

Breast cancer (BC) is one of the primary health problems worldwide (1, 2). According to the report of Non-Emmunicable Diseases Research Center of Iran, BC is the most prevalent cancer among the women in Kermanshah, West of Iran (33.02 new cases per 100000 women, in 2016) (3).

Early detection is critical for reducing the mortality and improving prognosis of this malignancy. Blood-based testing is recommended for detecting cancer biomarkers due to its easiness and lower invasiveness (4). However, measuring conventional serum biomarkers for BC detection, such as carbohydrate antigens (CA125 and CA15-3) and carcinoembryonic antigen (CEA) are not convincing in early detection (5). For this reason, new biomarker detection seems to be necessary for early diagnosis and monitoring of BC.

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By definition, long non-coding RNAs (lncRNAs) are biomolecules which their length is longer than 200 nucleotides. They can act as oncogenes or tumor suppressor genes, and their aberrant expression is probably involved in the progression of tumors (6). Linc00152, known as a cytoskeleton regulator long non-coding RNA (CYTOR), is an 828 bp gene located on the human chromosome 2p11.2 (7). CYTOR is up-regulated in various cancer types, including all subtypes of BC (8). Furthermore, recently it has been indicated that CYTOR could act as a circulating biomarker for the esophageal squamous-cell carcinoma, gastric cancer, and non-small-cell lung cancer (9-11). However, the potential clinical value of circulating CYTOR (c.CYTOR) in BC has not been investigated. Therefore, the aim of the present investigation was to study the levels of c.CYTOR in plasma of BC patients to show its role as a novel BC biomarker.

Materials and methods

Participants and sample collection

The BC cases were recruited at the time of the diagnosis, between 2016 and 2017 at the Bistoon Hospital, Kermanshah Province, Iran. The mean age of patients was about 49 years (range 30–68 years), and the mean age of healthy controls was 48 years (range 26–64 years, P = 0.14). The study was approved by the Ethics Committee of Kermanshah University of Medical Sciences and informed consent was obtained from all the subjects. Only new confirmed BC cases without a previous diagnosis of any malignant disease and the manifestation of any acute disease or injury were included in the present study. The first group consisted of tumoral and paired adjacent non-tumoral frozen tissues from 20 BC cases at the time of diagnosis. The second group consisted of 40 BC patients who were recruited only for peripheral blood sampling during diagnosis (before surgery and any therapy). Besides, a control group included 40 healthy blood donors. All participants were women with a Kurdish ethnic background from Kermanshah Province, Iran.

Plasma samples collection

The whole peripheral blood samples were collected in 5 mL EDTA containing tubes, then centrifuged (3500xg for 20 min) within an hour after blood collection to achieve plasma separation. The cellular fraction of prepared blood was separated, and plasma samples were stored at −80 °C.

RNA extraction and complementary DNA (cDNA) synthesis

Fresh frozen tissues were powdered in liquid nitrogen, and then total RNA was extracted by Qizol (QIAGEN, Germany) according to the manufacturer’s procedure. In addition, total RNA was isolated from 200 μL of plasma samples by using miRNeasy Plasma/Serum Kit (QIAGEN, Germany), and it was eluted into 14 μL of elution solution. The concentration and purity of the extracted RNA were evaluated by the Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). Complementary DNA (cDNA) was synthesized from the isolated total RNA by applying a QuantiTect Reverse Transcription Kit (QIAGEN, Germany).

Quantitative real-time PCR (qRT-PCR)

The sequences of selected primers used for qRT-PCR were 5’CTGGATGGTGCTGCTTCTT3’ (sense) and 5’GATCTGAAGACAGGGC-ACGGG3’ (antisense) for CYTOR (8), and 5’CTCGCTTCGCGACACA3’ (sense) and 5’AACGCTTCAGAATTGCAGT3’ (antisense) for U6-snoRNA (12) as an internal control. The qRT-PCR assay was performed using the Rotor-gene (Corbett Research, Germany) with SYBR Premix Ex Taq II (Takara, China). The PCR reaction conditions were initial denaturation at
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95 °C for 30 s, followed by 45 PCR cycles at 95 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s. We used the $2^{-\Delta\Delta Ct}$ method to determine the relative quantitation (RQ) in the cancerous samples relative to the control samples.

**Measurement of CA 15-3 in plasma**

The CA15-3 was assessed in accordance with the manufacturer’s protocol and reference intervals. This conventional tumor marker was measured in 80 plasma samples (40 healthy controls and 40 BC patients) using an ELISA kit (Monobind, USA). The CA15-3 threshold value provided by the company was equal to 37 U/ml. For plasma tumor marker definition, when its level exceeded a threshold value, the samples were considered to be positive.

**Statistical analysis**

Comparisons between groups were analyzed with Student’s t-test and $X^2$ tests. Statistical analyses were performed using SPSS (v.16.0) software. A P-value less than 0.05 was considered significant. Receiver operating characteristic (ROC) curves and the area under the curve (AUC) were used to assess the feasibility of using plasma CYTOR as a diagnostic tool for BC detection.

**Results**

The results of immunohistochemical analysis for hormone-responsiveness markers among 40 BC patients revealed 31 (77.5%) cases with estrogen (ER)-positive, 28 (70%) cases with progesterone (PR)-positive, 19 (47.5%) cases with HER2-positive, and 40 (100%) cases with Ki67-positive markers. Moreover, pathological examination reports of BC samples indicated the frequencies of 27.5%, 47.5%, and 25%, for stages I, II, and III, respectively.

**The expression level of CYTOR was significantly higher in tumoral tissues**

Using qRT-PCR, the relative expression level of CYTOR was measured in 20 breast tumor tissues and their adjacent non-tumoral tissues. The level of CYTOR gene expression in tumor tissues was significantly higher in comparison with paired adjacent non-tumoral tissues (P<0.001, Figure 1A).

The average RQ of tumor tissues in comparison with adjacent non-tumoral tissues was 5.15. There was no significant correlation between the tumoral tissue expression of CYTOR and the status of hormone-responsiveness markers (ER, PR, or HER2) or

![Fig.1. Relative expression of CYTOR. A: CYTOR expression in 20 paired breast tumor tissues and their adjacent non-tumoral tissues; B: relative expression levels of CYTOR in plasma of breast cancer patients in comparison with healthy controls.](image-url)
The plasma levels of *c.CYTOR* was significantly higher in BC patients

The relative level of circulating *CYTOR* expression was determined in each plasma sample. We found that the expression level of *CYTOR* in plasma samples collected from BC patients was significantly higher than the cancer-free donors (RQ= 3.03, \( P < 0.01 \), Figure 1B). This excess lncRNA *CYTOR* in the patient’s plasma is probably released from the tumor. Also, the analysis showed the absence of a significant correlation between the *c.CYTOR* levels and the status of hormone-responsiveness markers (ER, PR, or HER2) or tumor stages.

**Analysis of *c.CYTOR* stability in plasma**

To analyze the lncRNA stability in plasma, three plasma samples were divided into 4 equal parts, and incubated at room temperature for 0, 6, 12, and 24 h. Through defined time, no
considerable variation was observed in plasma CYTOR levels (P<0.05, Figure 2). These results indicated that CYTOR remained stable in plasma for at least 24 h, even at room temperature.

**Diagnostic value of c.CYTOR and CA 15-3 for BC**

To evaluate the potential clinical application of c.CYTOR, the ROC curve was constructed according to 80 BC patients and cancer-free controls data. The AUC of CYTOR was 0.907 (95% CI: 0.842–0.972, P<0.001, Figure 3A) for distinguishing BC patients from controls. Furthermore, for the comparison of the diagnostic value of c.CYTOR with a conventional plasma biomarker, cancer antigen 15-3 (CA 15-3) levels were assessed in 80 plasma samples (AUC= 0.868, 95% CI: 0.783–0.952, P<0.001, Figure 3B).

**Discussion**

Regarding the stability of circulating IncRNAs in plasma/serum, and their transcripts as the final functional products, assessing the expression levels of an IncRNA could directly show the levels of an active molecule. Accordingly, IncRNA transcripts have a potential value as a candidate for a non-invasive clinical index (13).

The present study was designed to assess the expression pattern and diagnostic value of IncRNA CYTOR in BC. First, we investigated the expression levels of CYTOR in BC tissues in comparison with adjacent non-tumoral tissues. We showed that CYTOR levels were higher in breast tumors in comparison with adjacent non-tumoral tissues. These results were in accordance with the previous studies, which suggested that CYTOR may play an oncogenic role in BC (8, 14-16).

Since it can be speculated that IncRNAs in circulation are unstable, thus, we then attempted to assess the stability of CYTOR in circulation. The results disclosed that CYTOR levels remained stable in plasma, which was consistent with previous studies (9, 11). Afterwards, we further found that c.CYTOR levels were significantly higher in the plasma of BC patients in comparison with healthy individuals, suggesting that CYTOR could offer a relatively high diagnostic efficacy for BC. The AUC of ROC curve for c.CYTOR was higher than CA 15-3 which is one of the conventionally used BC biomarkers (17, 18).

From eight reported articles related to the expression of c.CYTOR in plasma/serum, only one reported no significant differences between gastric cancer patients and normal controls (19), while others reported the upregulation of c.CYTOR in gastric cancer (10, 20, 21), hepatocellular carcinoma (22, 23), esophageal squamous-cell carcinoma (9), and non-small cell lung cancer (11). The results of our study, for the first time, suggested that CYTOR could be a circulating biomarker for BC diagnosis.

CYTOR has been introduced as an oncogenic IncRNA in different types of cancers, including tongue squamous cell carcinoma, pancreatic cancer, lung cancer, gastric cancer, liver cancer, and BC (16, 24-28). The molecular mechanism of CYTOR oncogenesis is not well understood, yet. It is shown that Yin Yang 1 (YY1), as a transcription factor, binds to the promoter of CYTOR and suppresses its transcription; while transcription Factor 3 (TCF3) could activate the transcription of CYTOR (16, 29). On the other hand, it was demonstrated that CYTOR overexpression promotes tumor cell cycle progression, cell viability, invasion, and epidermal-mesenchymal transition (EMT) (7, 8, 14, 30, 31).

Wu et al. (2018) showed that CYTOR knockdown promotes BRCA1/ phosphatase and tensin homolog (PTEN), expression through DNA methyltransferases (14). While Shen et al. (2018) demonstrated that knockdown or overexpression of CYTOR had no significant influence on the mRNA level of PTEN; instead, CYTOR impairs PTEN protein stability through triggering ubiquitination.
and degradation of PTEN protein by E3 ligase neural precursor cell expressed developmentally downregulated protein 4-1 (NEDD4-1) (16). PTEN acts via its phosphatase protein, which is involved in the cell cycle regulation and prevention of uncontrolled cell division (32).

CYTOR through the epidermal growth factor receptor (EGFR)-dependent pathway could promote cell viability (33) and through binding to the enhancer of zeste homolog 2 (EZH2) and consequently repressing p15 and p21 triggers cell cycle progression in gastric tumor (34). Furthermore, CYTOR through EGFR/PI3K/ATK pathway was shown to support cell viability and invasion in lung cancer (35). Some studies indicated that CYTOR can promote EMT in gastric cancer cells, liver cancer cells, and BC cells (28, 30, 36). EMT is a cellular process that is involved in tumor metastasis. In liver cancer cells, CYTOR could promote EMT by interacting with EZH2 and suppression of E-cadherin (28).

Totally, it seems that CYTOR through activation of EGFR signaling and suppression of PTEN and E-cadherin promotes EMT, which plays an important role in BC progression.

In conclusion, the findings of the present study suggest a role for plasma levels of IncRNA CYTOR as a candidate biomarker for BC that its level significantly increases in plasma of patients affected with BC. However, the biomarker role of this IncRNA needs to be investigated in other populations.

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Conflict of interest
Authors declare no conflict of interest in this study.

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