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Original Article

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-Eommunicable 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 clinic 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 patientsto 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 (3500×g 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'CTGGATGGTCGCTGCTTTTT3' (sense) and 5'GATCTGAAGACAGGCACGGG3' (antisense) for *CYTOR* (8), and 5'CTCGCTTCGGCAGCACA3' (sense) and 5' AACGCTTCACGAATTTGCGT3' (antisense) for U6-snRNA (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

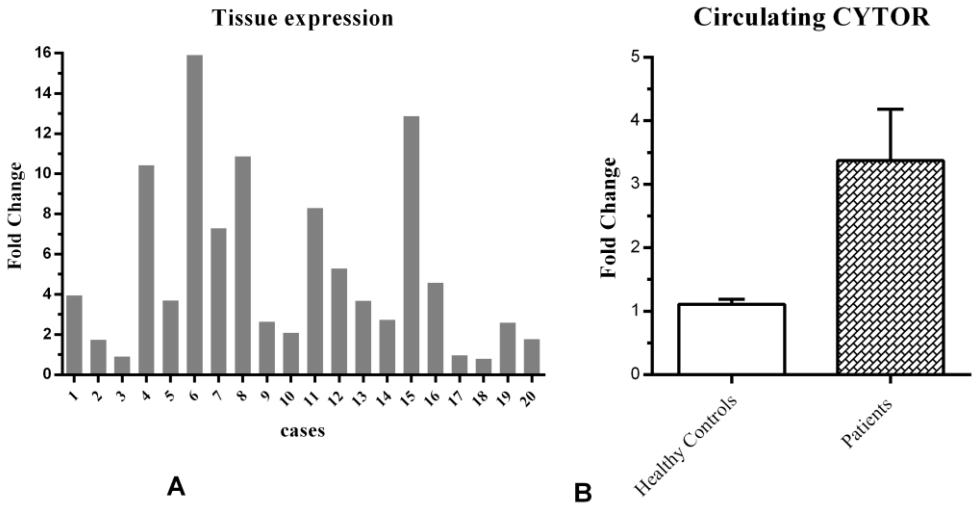

Fig.1. Relative expression of *CYTOR*. A: *CYTOR* expression in 20 paired breast tumor tissues and their adjacent non-tumoral tissues; B: relative expression levels of *CYTOR* in plasma of breast cancer patients in comparison with healthy controls.

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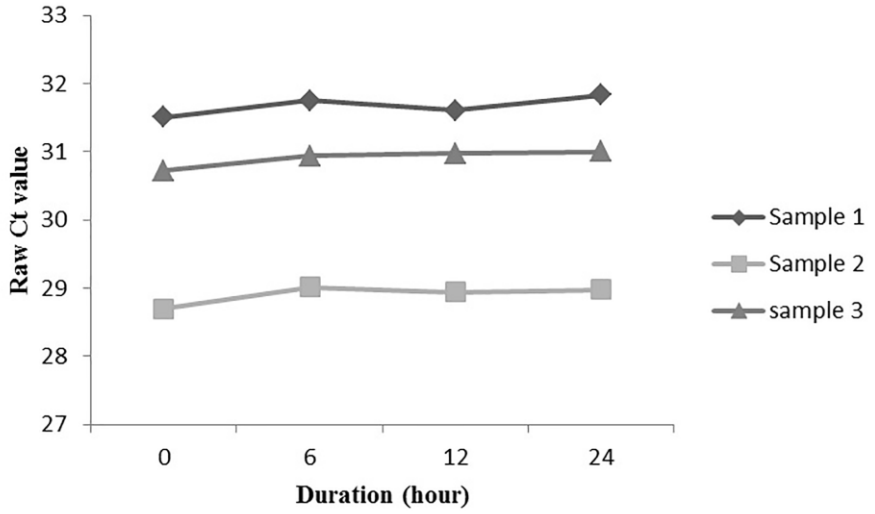

Fig.2. Stability assessment of circulating *CYTOR* in plasma. Three plasma samples were assessed for *CYTOR* stability right after sampling (0 h), after 6, 12, and 24 h incubation of plasma at room temperature. Ct: cycle threshold.

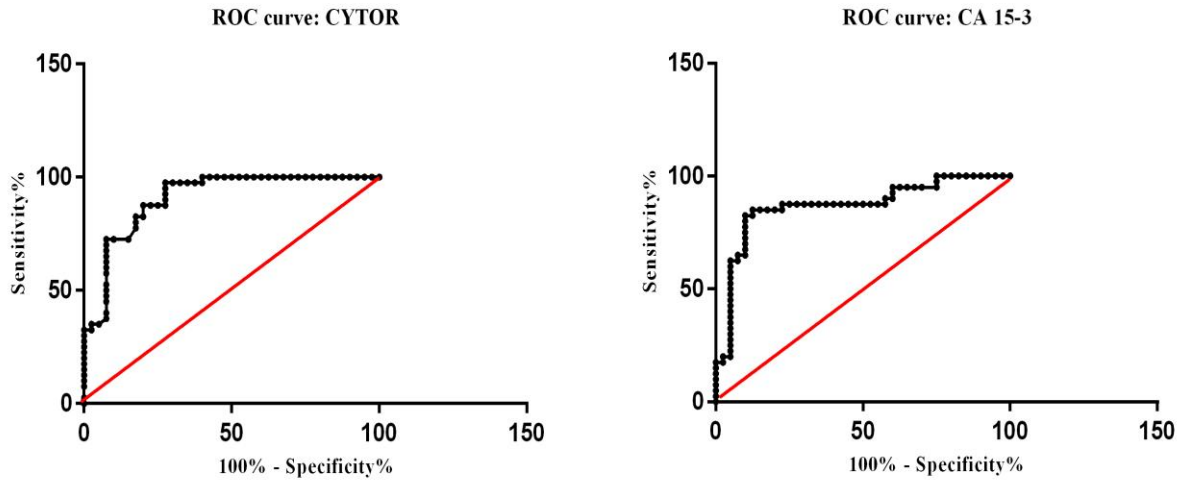

Fig.3. ROC curve alnaysis of *CYTOR* and CA 15-3 in plasma of breast cancer patients. A: evaluation of the diagnostic value of circulating *CYTOR*; B: CA 15-3 diagnostic value.

tumor stages.

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

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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 lncRNAs in plasma/serum, and their transcripts as the final functional products, assessing the expression levels of a lncRNA could directly show the levels of an active molecule. Accordingly, lncRNA 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 lncRNA *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 lncRNAs 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 lncRNA 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

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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 lncRNA *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 lncRNA 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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