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Upregulation of CircCSPP1, CircNRIP1, and CircSMAD2 in Breast Cancer and Their Potential as Diagnostic Biomarkers

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ABSTRACT

Background: Identifying novel diagnostic biomarkers with stable structures, such as circRNAs can improve the early detection and management of BC. Herein, we conducted this study to analyze the expression profile of four circRNAs, including circCSPP1 (hsa_circ_0001806), circNRIP1 (hsa_circ_0004771), circSMAD2 (hsa_circ_0000847), and circFOXP1 (hsa_circ_0008234) in BC.

Methods: Tumor tissues and adjacent normal tissues were obtained from patients with sporadic BC. Divergent primers were designed to amplify the target transcripts using quantitative real-time PCR. The expression profiles of circRNAs were analyzed in tumor and adjacent normal tissues. Sanger sequencing was performed to confirm the back-splicing junctions of circRNAs. ROC curves were generated to assess the potential of the mentioned RNAs as diagnostic biomarkers.

Results: We observed a significant upregulation of circCSPP1, circNRIP1, and circSMAD2 in tumor tissue compared to adjacent normal tissues. Among them, circCSPP1 was the most highly upregulated one in tumor samples from 39 patients. Expression of circCSPP1 was significantly higher in ER-negative, PR-negative, HER2-negative, and triple-negatives compared to ER-positive, PR-positive, HER2-positive, and non-triple-negative ones. Expression of circNRIP1 was also significantly elevated in the ER-negative and the triple-negative subgroups. These three circRNAs also displayed a desirable potential as diagnostic biomarkers.

Conclusion: This is the first paper that reports the upregulation of circCSPP1 and circSMAD2 in BC and upregulation of circCSPP1 and circNRIP1 in the triple-negative subgroup. Our findings suggest that circCSPP1, circNRIP1, and circSMAD2 may serve as promising diagnostic biomarkers for BC. Identifying the downstream pathways regulated by these circRNAs could lead to the discovery of new therapeutic targets. ***DOI: 10.61882/ibj.4945***

Keywords: Breast neoplasms, Circular RNA, Triple negative breast neoplasms, Tumor biomarkers

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List of Abbreviations:
AUC: area under the curve; **BC:** breast cancer; **cDNA:** complementary DNA; **circRNA:** circulating circular RNA; **CSPP1:** centrosome and spindle pole-associated protein 1; **EMT:** epithelial-to-mesenchymal transition; **ER:** estrogen receptor; **FOXP1:** forkhead box protein P1; **HER2:** human epidermal growth factor receptor-2; **mRNA:** messenger RNA; **NRIP1:** nuclear receptor-interacting protein 1; **PR:** progesterone receptor; **ROC:** receiver operating characteristic; **SMAD2:** decapentaplegic homolog 2; **TNBC:** triple-negative breast cancer; **TNBC:** triple-negative breast cancer; **ZEB2:** zinc finger E-box binding homeobox 2

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INTRODUCTION

Breast cancer was the second commonly diagnosed malignancy worldwide in 2022, with 2.31 million new cases. It remains the leading cause of cancer-related mortality in women[1]. Reducing the BC mortality relies on early detection, as well as precise and effective treatments. One key strategy to achieve this objective is the identification of novel biomarkers. CircRNAs are important biomarkers that facilitate the diagnosis and treatment of cancer[2]. They are endogenous non-coding RNA molecules characterized by a closed-loop structure formed through the back-splicing of the precursor mRNAs. Back-splicing occurs by conjunction of the 3′ splice donor sequence into the upstream 5′ splice acceptor sequence[3]. CircRNAs are suitable biomarkers due to their stable structure compared to other RNA molecules, and their expression patterns that are associated with different developmental stages[4].

CircRNAs contribute to the development and progression of BC through various mechanisms. For instance, circGFRA1 promotes tumor cell proliferation and clonogenicity[5], circIRAK3 enhances migration and invasion[6], and circANKS1B drives EMT and metastasis[7]. CircRNAs perform these roles through various mechanisms, where the majority act as microRNA sponges, competing with mRNAs[8,9]. They also alter RNA-binding proteins, directly regulate gene expression, and compete with linear mRNAs for translation[9]. These functions primarily serve to regulate gene expression.

In the present study, we selected four circRNAs with a role in cancer progression to evaluate their expression profiles in BC. Among these RNAs, circNRIP1, derived from the *NRIP1* gene, had been previously investigated in BC[10]. The other three circRNAs had not been studied in BC, although their roles have been explored in other types of cancer. The three RNAs include circCSPP1 derived from the *CSPP1* gene, circSMAD2 derived from the *SMAD2* gene, and circFOXP1 derived from the *FOXP1* gene. In this study, we analyzed the expression patterns and diagnostic potential of these circRNAs in BC tissues compared to normal breast tissues.

MATERIALS AND METHODS

Patients and samples

We recruited patients with sporadic BC who were referred to Shahid Faghihi Hospital, Shiraz, Iran, for BC surgical excision. The exclusion criteria were familial history of either BC or ovarian cancer. Patients who received chemotherapy, radiotherapy, or immunotherapy before surgery, were also excluded. The required data were extracted from the dataset GSE101124. With power of 80% and 95% CI, a sample size of 41 was achieved.

$$n = \frac{\left(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta}\right)^2 (\delta_1^2 + \delta_2^2)}{(\mu_1 - \mu_2)^2}$$

Matched tissues, including tumor and adjacent normal tissue samples, were obtained from the participants. In addition, 10 normal mammary tissues were obtained from healthy individuals without a family history of BC who had undergone cosmetic mammoplasty. These normal tissues were used as calibrator samples to achieve relative expression. We retrieved demographic characteristics, including age and clinicopathological data, and stratified patients according to these characteristics based on the cutoffs often used for categorizing BC patients[11] (Table 1).

Circular RNAs

We used Circular RNA Interactome (https://circinteractome.irp.nia.nih.gov/) and Circbank (https://www.circbank.cn/) databases and performed a literature review to find circRNAs that were upregulated in different cancer types and associated with worse clinicopathological characteristics in previous studies. We selected one circRNA that has formerly been studied in BC. circNRIP1 (hsa_circ_0004771), and three circRNAs, including circCSPP1 (hsa_circ_0001806), circSMAD2 (hsa_circ_0000847), and circFOXP1 (hsa_circ_0008234) have been previously studied only in other cancer types.

RNA extraction and cDNA synthesis

Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, USA) according to the manufacturer's instruction. The quality of the extracted RNA was confirmed by a Nanodrop 2000C spectrophotometer (Thermo Fisher. Scientific), and its integrity was assessed by 2% agarose gel electrophoresis. The cDNA synthesis was performed using the “PrimeScriptTM 1st Strand cDNA Synthesis Kit”, including oligo-dT and random hexamer (Takara Bio, Japan).

Quantitative real-time PCR

Divergent primers were designed to amplify the target transcripts of circRNAs by quantitative real-time PCR. CircPrimer 2.0 was used to design the primers. The forward and reverse primers (5’ ≥ 3’) used in this study were as follows: GTGTCTCCCAGTGCTCCAG and CAGCCATTTGTTCCAACAGTTC for circCSPP1, GCCAGAAGATGCACACTTGAC and CAAACAC TTCCGTCTGTCTCC for cir cNRIP1, TATGGACA

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Table 1. Log2 fold change of circRNAs in tumor tissues based on clinicopathological characteristics

Characteristic	Subgroup (frequency)	circCSSP1 (mean ± SD)	circNRIP1 (mean ± SD)	circSMAD2 (mean ± SD)	circFOXP1 (mean ± SD)
	<50 (18)	3.8 ± 1.6	3.6 ± 1.7	2.6 ± 1.1	1.5 ± 2.7
	≥50 (21)	4.2 ± 1.5	3.9 ± 1.8	2.7 ± 0.8	2.0 ± 2.2
	p	0.778	0.888	0.921	0.472
	<2.5 (21)	3.6 ± 1.5	3.7 ± 1.5	3.0 ± 0.8	2.0 ± 2.2
	≥2.5 (18)	4.5 ± 1.5	3.8 ± 2.1	2.3 ± 1.0	1.6 ± 2.7
	p	0.061	0.966	0.063	0.602
	Negative (12)	5.0 ± 1.5	4.8 ± 2.2	2.7 ± 0.9	2.1 ± 2.2
	Positive (27)	3.6 ± 1.4	3.3 ± 1.4	2.7 ± 1.0	1.7 ± 2.5
	p	**0.005**	**0.049**	0.867	0.692
	Negative (13)	5.0 ± 1.4	4.6 ± 2.1	2.7 ± 0.9	2.1 ± 2.1
	Positive (26)	3.5 ± 1.3	3.3 ± 1.4	2.7 ± 1.0	1.7 ± 2.6
	p	**0.001**	0.071	0.964	0.655
	Negative (28)	4.3 ± 1.6	3.9 ± 1.9	2.5 ± 0.9	1.5 ± 2.5
	Positive (11)	3.4 ± 1.1	3.4 ± 1.5	3.1 ± 1.0	2.7 ± 2.2
	p	**0.030**	0.673	0.180	0.095
	No (27)	3.6 ± 1.4	3.3 ± 1.4	2.7 ± 1.0	1.7 ± 2.5
	Yes (12)	5.0 ± 1.5	4.8 ± 2.2	2.7 ± 0.9	2.1 ± 2.2
	p	**0.005**	**0.049**	0.867	0.692
	Negative (23)	3.9 ± 1.5	3.9 ± 1.4	2.9 ± 0.8	2.4 ± 2.3
	Positive (16)	4.2 ± 1.6	3.6 ± 2.2	2.4 ± 1.1	0.9 ± 2.4
	p	0.898	0.077	0.130	0.061
	G1 (7)	4.6 ± 0.9	3.2 ± 1.4	2.4 ± 0.8	1.3 ± 2.3
	G2 (18)	3.9 ± 1.1	3.7 ± 1.2	2.9 ± 0.8	1.6 ± 2.1
	G3 (14)	3.9 ± 2.2	4.2 ± 2.4	2.5 ± 1.2	2.3 ± 2.9
	p	0.569	0.324	0.350	0.741

Kruskal-Wallis H test was used for comparison of histologic grade subgroups, while Mann-Whitney U test was used for the other analyses. Bold values show significance at $p < 0.05$ level.

CAGGTTCGATACAAG and CTCCTCCAGACCCA CCAGcirc for circSMAD2, CCCAAAAGGGAAA GGTTCC and CTGCTGGAGGAGAACCTG for FOXP1, and AGTGGGGGACTAGGCGTTAG and GTTTTCATCACTGTCTGCATCC for PUM1. The relative expression of circRNAs was evaluated in duplicate using RealQ Plus 2× Master Mix Green low ROX (Ampliqon, Denmark) on a 7300 Thermocycler (Applied Biosystems, Thermo Fisher Scientific). The *PUM1* housekeeping gene was used to normalize data. *PUM1* is the most stable housekeeping gene used in the studies on breast tissue[12]. The reaction conditions for all circRNAs and the housekeeping gene, except for circFOXP1, consisted of 15-minute preincubation at 95 °C, followed by amplification in 35 cycles at 95 °C for 15 seconds and then 59 °C for 45 seconds. The annealing temperature for circFOXP1 was 60 °C. The $2^{-\Delta\Delta CT}$ method was applied for the calculation of relative expression. To confirm the amplification and integrity of circRNAs, we used 2% agarose gel electrophoresis and Sanger sequencing in order to verify the back-splicing junction and the circular structure of circRNAs. For Sanger sequencing, the primers that contained the back-splicing junctions were selected as follows: the forward primers for circCSPP1, circSMAD2, circFOXP1 and the reverse primers for circNRIP1. SnapGene 5.3.1 was used for the analysis of Sanger sequencing data.

Statistical analysis

We used the SPSS Statistics software 22 and GraphPad Prism 8 for data analysis and visualization. The Heatmap, ROC curve, and other charts were created by GraphPad Prism 8. Continuous variables were assessed through the Kolmogorov-Smirnov test to check normality. Based on the distribution of variables, the nonparametric Wilcoxon Signed Ranks test was applied to compare the relative expression of circular RNAs in the tumor and adjacent normal samples. For the comparison of subgroups, the Mann-Whitney U test and

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Kruskal-Wallis test were used. The statistical significance level was considered at $p < 0.05$.

RESULTS

We included 41 women with invasive BC, and samples from 39 cases were included in the analysis. Samples from two patients were excluded due to a problem in the RNA extraction step.

Expression profile of circRNAs in breast tumor and normal tissues

Three of the four circRNAs studied, i.e. circCSPP1, circNRIP1, circSMAD2 (all with $p < 0.0001$), displayed a statistically significant upregulation in tumor tissues compared to adjacent normal tissues (Fig. 1). CircFOXP1 was also upregulated in tumor tissues; however, it was not statistically significant ($p = 0.08$). The quantitative real-time PCR was performed, and the PCR products were evaluated by Sanger sequencing. The selected primers, which targeted the back-splicing junctions of the studied circRNAs, confirmed the presence of these junctions through Sanger sequencing. The back-splicing junctions of the circRNAs were identified as follows: circCSPP1 at the junction of Exon 11-8 (Fig. 2A), circNRIP1 at the junction of Exon 3-2 (Fig. 2B), circSMAD2 at the junction of Exon 5-2 (Fig. 2C), and circFOXP1 at the junction of Exon 11-8 (Fig. 2D).

Expression profile of circRNAs in different subgroups

Our study revealed an association between the expression level of circRNAs in tumor tissues and clinicopathological characteristics of the patients (Table 1). The ER-negative tumor samples, compared to the ER-positive ones, exhibited increased expression of circCSPP1 ($p = 0.005$) and circNIRP1 ($p = 0.049$). The PR-negative tumor samples, compared to the PR-positive tumors, displayed an increased expression of circCSPP1 ($P = 0.001$). Interestingly, HER2-negative tumor samples also displayed higher expression of circCSPP1 ($p = 0.030$) compared to HER2-positive ones. However, the differential expression of circNRIP1 between PR-negative and PR-positive tumor samples and between HER2-negative and HER2-positive tumor samples was not statistically significant. Upregulated expression of the circCSPP1 ($p = 0.005$) and circNIRP1 ($p = 0.049$) was also observed in the TNBC subgroup compared to the non-TNBC subgroup (Table 1). There was no significant association between the expression level of circCSPP1 and circNIRP1 and other clinicopathological characteristics. The expression level of circSMAD2 and circFOXP1 were not also correlated with clinicopathological characteristics.

CircRNAs as diagnostic biomarkers

The four studied circRNAs were evaluated as diagnostic biomarkers by creating ROC curves. The analysis indicated that three of these RNAs might serve as diagnostic biomarkers for BC. The AUCs for circNRIP1, circCSPP1, and circSMAD2 were respectively 0.98, 0.95, and 0.91, with p value less than 0.0001 (Fig. 3). However, circFOXP1 did not show any significant diagnostic value. Among the circRNAs, circCSPP1 was the most sensitive diagnostic biomarker with a sensitivity of 0.95 (95% CI: 0.83-1.00), whereas circNRIP1 and circSMAD2 were the most specific biomarkers, both sharing a specificity of 0.97 (95% CI: 0.87-1.00). CircNRIP1, compared to circSMAD2, demonstrated a superior sensitivity (0.92 vs. 0.82) and likelihood ratio (36.0 vs. 32.0). Therefore, the ideal diagnostic biomarker was circNRIP1, with the AUC of 98%, sensitivity of 92%, specificity of 97%, and the likelihood ratio of 36.0.

DISCUSSION

The use of biomarkers for diagnosis, prognosis, and predicting treatment response represents a significant advancement in the timely management of BC[13]. Dysregulated circRNAs are emerging biomarkers that have been investigated for the diagnosis and prognosis of various cancers. CircCSPP1 is a key epigenetic regulator that has experimentally been validated. In this context, Wang et al. reported its upregulation in colon cancer tissues, where it promotes tumor cell proliferation, migration, and invasion[14]. Its knockdown suppresses tumorigenesis, primarily through sponging miR-431. Two additional studies highlighted the role of circCSPP1 in upregulating COL1A1, which contributes to the stemness of colorectal cancer cells[15] and promotes their metastasis to the liver[16].

In the current study, we observed a significant upregulation of circCSPP1 in breast tumor samples compared to normal samples. Additionally, it exhibited a notable upregulation in the ER-negative, PR-negative, HER2-negative, and TNBC subgroups. This finding suggests an important role for circCSPP1 in BC, particularly in TNBC. It is important to note that in our study, the ER-negative subgroup overlapped the TNBC subgroup. When interpreting results based on ER status, this overlap should be taken into account. The non-TNBC subgroup consisted of both PR-negative and HER2-negative samples but no ER-negative cases; therefore, our findings primarily reflect the TNBC

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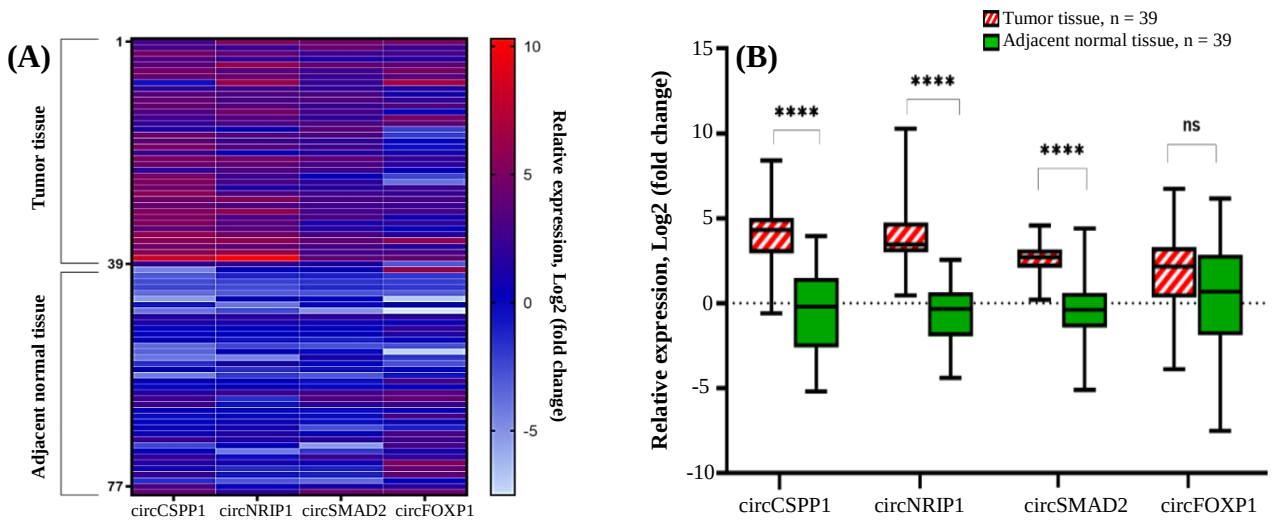

Fig. 1. Relative expression of circRNAs in tumor tissues and adjacent normal tissues. The Log2 fold change was used to illustrate the differential expression of circRNAs. (A) the heatmap displayed the expression level of circRNAs in each sample; (B) comparison of circRNA expression in tumor and adjacent normal tissues. Three circRNAs, including circCSPP1 ($p < 0.0001$), circNRIP1($p < 0.0001$), and circSMAD2 ($p < 0.0001$) demonstrated statistically significant expression in tumor tissues compared to matched adjacent normal tissues. ns: not significant

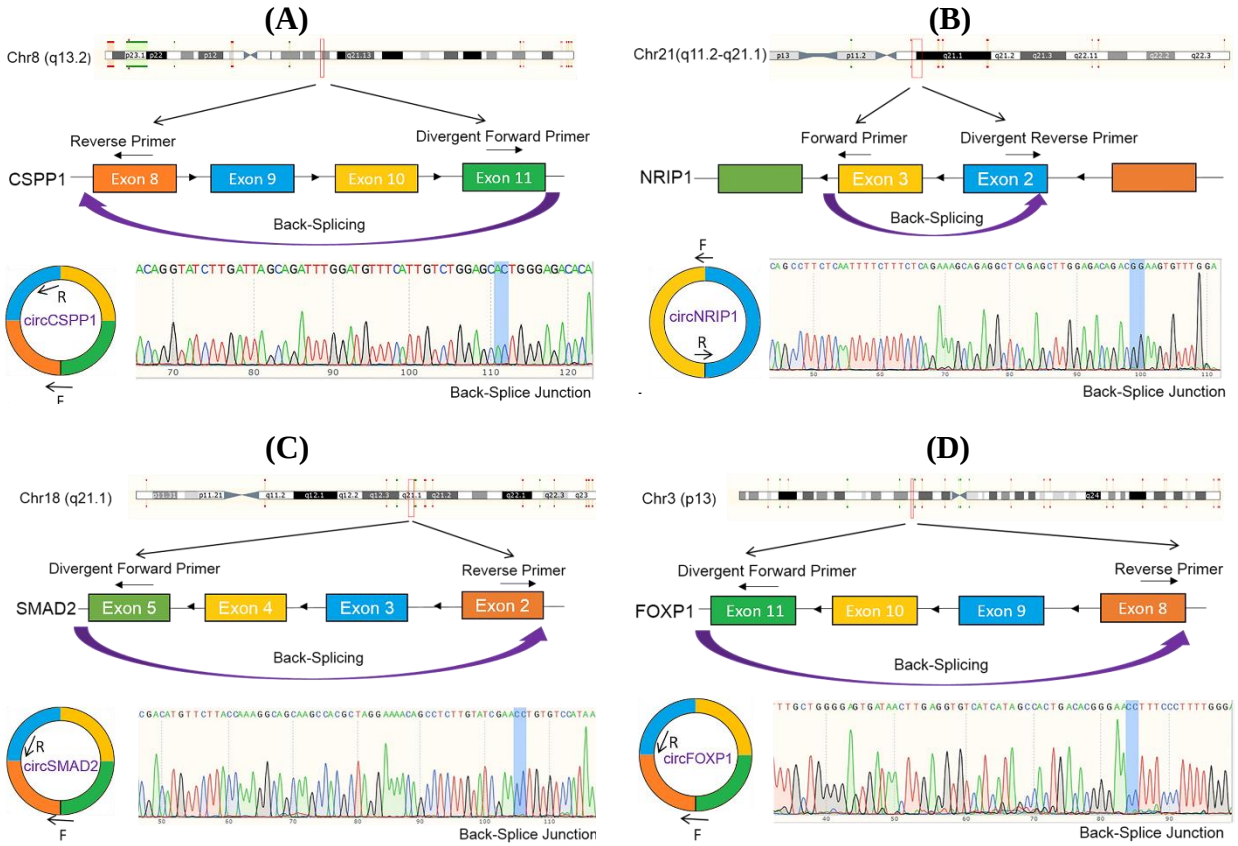

Fig. 2. Schematic presentation of circRNAs and verification of back-splicing junctions by Sanger sequencing. (A) circCSPP1; (B) circNRIP1; (C) circSMAD2; (D) circFOXP1.

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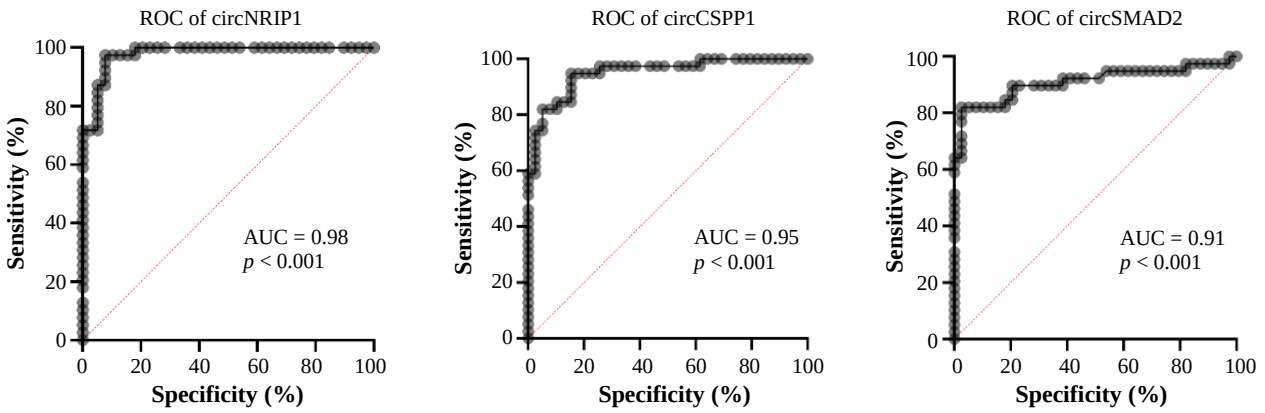

Fig. 3. ROC curves of circRNAs.

samples. As a diagnostic biomarker, circCSPP1 demonstrated strong potential in our study with an ideal AUC, high sensitivity, and excellent specificity. It may also warrant further investigation as a biomarker in TNBC.

CircNRIP1 is involved in the proliferation, migration, and invasion of tumor cells[10,17-19]. An upregulation in the circNRIP1 expression level has been reported in cervical cancer[17], gastric cancer[18], esophageal cancer[19], and BC[10,11]. In a study by Xie et al., circNRIP1 was identified as the most significantly upregulated circRNA in BC among five candidates selected through high-throughput sequencing[10]. CircNRIP1 is associated with poor prognosis and promotes BC progression by sponging miR-653 and upregulating ZEB2, a transcription factor involved in EMT. The knockdown of circNRIP1 and ZEB2 induced apoptosis in tumor cells, While Xie et al. did not analyze circNRIP1 expression level across different BC subgroups[10]. In our analysis, we found a significant upregulation of circNRIP1 in the ER-negative and the TNBC subgroups. In our study, circNRIP1 displayed excellent potential as a diagnostic biomarker with an ideal AUC, sensitivity, and specificity. It may serve as a strong, sensitive, and specific biomarker to facilitate the diagnosis of BC.

Previous studies have reported the upregulation of circSMAD2 in gallbladder cancer[20] and also advanced colorectal cancer[21], highlighting its role in promoting tumor cell proliferation, migration, and invasion, as well as its association with metastasis and tumor stage[21]. In contrast, another study found circSMAD2 to be downregulated in poorly differentiated hepatocellular carcinoma, where it inhibits migration, invasion, and EMT by sponging miR-629[22]. These conflicting findings indicate that circSMAD2 may exhibit both pro-tumorigenic and anti-tumorigenic roles. In the present study, we observed a significant upregulation of circSMAD2 in breast tumor samples. Also, it demonstrated a strong potential as a diagnostic biomarker. Similar to circNRIP1, circSMAD2 ranked first in diagnostic specificity highlighting its specific ability for breast cancer diagnosis. Luo et al. revealed that circFOXP1 promotes tumor cell proliferation in colorectal cancer by inhibiting FOXP1 expression through promoter methylation. Its upregulation in colorectal cancer tissues was associated with advanced disease stages[23]. Another study by Wang et al. reported the upregulation of circFOXP1 in gallbladder cancer and its association with lymph node metastasis, advanced stage, and poor clinical prognosis. They also demonstrated that circFOXP1 facilitates proliferation, migration, invasion, and inhibiting apoptosis of tumor cells by binding to PTBP1 and/or sponging miR-370 to regulate PKLR[24]. In contrast, a recent study reported its downregulation in lung adenocarcinoma, exhibiting an anti-tumorigenic effect by sponging miR-574-5p[25]. These findings suggest that circFOXP1 plays a dual role depending on its downstream target. In our study, circFOXP1 was upregulated in tumor tissue samples compared to normal tissues; however, this difference was not statistically significant. It may also warrant further investigation as a pro-tumorigenic biomarker in BC.

CONCLUSION

Our study is the first to evaluate the expression of circCSPP1, circSMAD2, and circFOXP1 in this context. Herein, we observed a significant upregulation of circCSPP1, circNRIP1, and circSMAD2; among them, circCSPP1 was the most highly upregulated circRNA. The AUC, sensitivity, and specificity of the ROC curve for circCSPP1, circNRIP1, and circSMAD2 showed their significant potential as diagnostic biomarkers for

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BC. Additionally, circNRIP1 exhibited significant upregulation in the ER-negative and TNBC subgroups. Given that TNBC is associated with a poor prognosis, identifying novel pathways involved in tumorigenesis and invasion of this subtype is of great importance. For the first time, we found that circCSPP1 and circNRIP1 are significantly upregulated in TNBC. Investigating the downstream miRNAs and target molecules regulated by these circRNAs may reveal new therapeutic targets for TNBC. We acknowledge the limitation of not assessing expression patterns in plasma samples. Future studies, particularly those focusing on TNBC, are needed to address this gap.

DECLARATIONS

Acknowledgments

This paper is dedicated to Mrs. Fatemeh Sinaei who passed away because of breast cancer. We acknowledge that we did not use artificial intelligence (AI)-assisted technologies in the production of this work. We received some suggestions for English editing of the manuscript from ChatGPT; however, we did not apply most of them.

Ethical approval

The procedures of this study was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (ethical code: IR.TUMS. CHMC.REC.1400.058).

Consent to participate

Written informed consent for publication was obtained from all participants.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

MKF: lab experiments, data analysis, visualization, and manuscript preparation; FD: study design and lab experiments; RM, PSP, and HS: lab experiments and study design; YM: samples collection and experimental studies; AMM: study design and supervision; NR: study design, supervision, and funding acquisition.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

Competing interests

The authors declare that they have no conflict of interest.

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Supplementary information

The online version does not contain supplementary material.

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