Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 3621 - 3636
Received 25 April 2021; Accepted 08 May 2021.

Expression Profiles of Novel Breast Cancer- Associated lncRNAs in Relation to Tumor Characteristics and Reproductive Factors

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ABSTRACT

Background: Previous studies revealed important functions of three lncRNAs FAL1, HAND2-AS1, and PTCSC3 in development of various cancers; however, their roles in breast cancer is currently unknown.
Materials and methods: In the present study, expression levels of LncRNAs were detected by qPCR in 50 breast- tumor and tumor adjacent normal tissues (TANTs).
Results: In this study, we found that lncRNA FAL1 was upregulated, while lncRNAs HAND2-AS1 and PTCSC3 were downregulated in tumor tissues than in TANTs (P values < 0.001). Moreover, the results of the current study revealed higher expression of FAL1 in tumors with larger sizes (P = 0.003), higher nuclear grades (P < 0.001), as well as negative statuses of estrogen receptor (ER), progesterone receptor (PR), and HER2 (P < 0.001, P = 0.001 and P < 0.001 respectively). Regarding to HAND2-AS1, our results showed upregulation of this lncRNA in smaller tumor sizes and lower nuclear grades (P values < 0.001). Furthermore, we observed higher levels of PTCSC3 in tumors with $HER2^-$ status (P = 0.012), free-lymph node metastasis (LNM) and early stages of BC (P values < 0.001). Finally, our results showed significant differences in expression levels of these lncRNAs between subgroups of some reproductive characteristics including breastfeeding duration, parity, and menopause status.
Conclusion: Therefore, lncRNA FAL1 may has oncogenic role in BC and its expression levels correlate with worst outcomes of disease. Inversely, lncRNAs HAND2-AS1 and PTCSC3 may have anti- tumorigenic roles in breast cancer.

Keywords
breast cancer; lncRNA; FAL1; HAND2-AS1; PTCSC3

Introduction

Breast cancer (BC) is the most frequently occurring malignancy in women and the leading cause of cancer related death among women worldwide (Bray and others 2018). This cancer shows a highly heterogeneous nature in terms of clinical outcomes and biological behavior (Rivenbark and others 2013; Weigelt and others 2010). The body of literature suggests that some phenotypic behaviors of different BC subtypes are due to the altered expression profiles of specific non-coding RNA (ncRNA) occurred during different stages of breast tumorigenesis and ultimately dictate the discrete tumor natures (Klinge 2018; Tian and others 2018a; Wang and others 2017a; Zhang and others 2016).Therefore, pinpointing the nature differences of such RNA signatures in the various clinical phases of the breast malignancy may provide better and more effective advances in the current procedures of prevention, diagnosis, and treatment of breast tumors. In recent years, progress in RNA sequencing techniques, epigenomic technologies, and computational prediction techniques has revealed that the human genome encodes over 30,000 different long noncoding RNAs (lncRNAs) transcripts as a class of ncRNAs (Clark and others 2015; Guttman and others 2009). The lncRNAs have longer than 200 nucleotides in length and do not encode proteins (Evans and others 2016).Owing to the heterogeneous and multi-functional roles of lncRNAs in regulating of gene expression, they are

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described as "master regulators" of transcriptome (Abdollahzadeh and others 2019; Chu and others 2011; Harries 2012; Khalil and others 2009).

Moreover, it has been revealed that lncRNAs play vital roles in tumorigenicity and clinical outcomes of different cancers, including BC (Qi and Du 2013; Tian and others 2018b; Vikram and others 2014) . Three important lncRNAs whose dysregulated expression play central roles in different tumor hallmarks including, tumor progression, metastasis, and prognosis of various cancers are FAL1, HAND2-AS1, and PTCSC3. Nevertheless, data on expression patterns of these key lncRNAs in BC have not yet been reported.

According to previous studies, lncRNA FAL1 had outstanding oncogenic activity in several types of cancers, such as thyroid cancer (Jeong and others 2016), non-small cell lung cancer (NSCLC) (Pan and others 2017), hepatocellular carcinoma (HCC) (Li and others 2018), gastric cancer (Zhu and others 2018), esophageal cancer cell (Liang and others 2018; Yang and others 2019), and colorectal cancer (CRC) (Wang and others 2019a; Wu and others 2018). In addition, it has displayed that upregulation of FAL1 correlates with advanced TNM stages, higher histological grades, positive lymph node metastasis, and poor prognosis (Li and others 2018; Liang and others 2018; Pan and others 2017; Wang and others 2019a; Wu and others 2018; Yang and others 2019; Zhu and others 2018).

LncRNA HAND2-AS1 is a recently identified lncRNA that serves as a tumor suppressor gene in various cancers, such as endometrial carcinoma (Yang and others 2018), CRC (Zhou and others 2018), esophagus squamous cell carcinoma (ESCC) (Miao and others 2019), non-small cell lung cancer (NSCLC) (Miao and others 2019), ovarian cancer (Chen and others 2019a), HCC (Wang and others 2019b), melanoma (Liu and others 2019), and Osteosarcoma (Chen and others 2019b; Kang and others 2018). Down-regulation of HAND2-AS1 was correlated with worse outcomes of disease, including higher tumor grade, presence of lymph node metastasis, and recurrence (Yang and others 2018), as well as advanced stages (Liu and others 2019; Miao and others 2019; Zhou and others 2018).

In the case of lncRNA PTCSC3, the results of all studies in various cancers indicate its tumor suppressive function (Fan and others 2013; Wang and others 2018; Wang and others 2017b; Xiao and others 2019). It has been revealed that lncRNA PTCSC3 can suppress tumorigenesis in glioma and thyroid cancer via Wnt/β-catenin signaling pathway (Wang and others 2017b), triple- negative BC (TNBC) through lncRNA H19 (Xiao and others 2019), and laryngeal squamous cell carcinoma (LSCC) through lncRNA HOTARIR (Xiao and others 2019).

Taken together, these research clues suggested that FAL1, HAND2-AS1, and PTCSC3 have critical roles in multiple malignancies and may serve as potential therapeutic targets for the cancer treatment. However, evidence behind the expression of these lncRNAs in BC malignancy as well as correlations of their expression patterns with the clinicopathology features of the breast tumors remain unclear. Thus, the leading objectives of the current study were to investigate the expression of three lncRNAs FAL1, HAND2-AS1, and PTCSC3 in breast tumors and tumor-adjacent normal breast tissues as well as exploring their possible expression correlations with the clinicopathology aspects of the tumors in patients.

Material and Methods

Tissue sampling

50 paired BC tissues and corresponding adjacent normal tissues were obtained from Imam Khomeini Hospital (Tehran, Iran). All tissue specimens were confirmed by pathologically and histologically examinations. Samples were immediately frozen in liquid nitrogen and stored at -80^0C until used. All of the clinicopathological, demographic, and reproductive characteristics of the patients have been recorded and analyzed. This study was

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conducted in accordance with the ethical principles of the World Medical Association's Declaration of Helsinki. Written informed consent was signed by all participants.

Expression analysis

Total RNA was extracted from BC tissues and tumor adjacent normal tissues (TANTs) using The RiboEx solution (GeneAll, South Korea) according to the manufacturer's protocol. To removing of the DNA contaminations, DNase (Catalog number: 2270A, Clontech, Japan) was applied. Abundance of intracellular RNA was determined by using a NanoDrop apparatus. The first-strand cDNA was synthesized from 0.5 µg of total RNA by using the PrimeScriptTM RT Reagent kit, according to the manufacturer-provided protocol (Cat. #: RR037A, Takara, Clontech, Japan). Quantitative RT-PCR (qRT-PCR) was performed for evaluating of the gene expression in duplicate on a Light Cycler® 96 Instrument (lifescience-roche, Germany). Each PCR reaction comprised of 5 µl RealQ Plus 2x MasterMix Green (Cat. #: A324406, Amplicon, Denmark), 1 µl cDNA (≥10 ng), 0.5 µl of each primer (5 pmol) and 3 µl of nuclease-free water in a total volume of 10 µl reaction mixture. Primers for amplification of studied genes were showed in table 1. Two-step PCR thermal cycling and real-time data acquisition were performed using the following conditions: 15 min at 95 °C (for primary denaturation), followed by 40 cycles of 10 s at 95 °C (denaturation) and 25 s at 60 °C (annealing and extension), followed by the melt curve stage assessment. To check the primer specificity, melting curves analysis and agarose gel (2%) electrophoresis was performed. The relative expression level of three lncRNAs was normalized to the expression level of housekeeping gene *β2M*.

Table 1. Sequence of primers used in present study

Genes	Primer type	Primer sequence
FAL1	F	CCTGGCCAAGAAGCTCATAC
	R	TGAGGACACCGACTACTGAGAA
HAND2-AS1	F	GGGTGTTTACGTAGACCAGAACC
	R	CTTCCAAAAGCCTTCTGCCTTAG
PTCSC3	F	GGCTTGAACAATCTTCCCACCTT
	R	TTTGGCAACACCCTCACAGACAC
B2M	F	AGATGAGTATGCCTGCCGTG
	R	GCGGCATCTTCAAACCTCCA
F: forward primer; R: reverse primer; RT: reverse transcription primer		

Statistical analysis

statistical analysis was carried out by IBM SPSS statistics version 19 (IBM SPSS Inc, Chicago, IL, USA). The One-Sample Kolmogorov-Smirnov test was used to determine whether data are normally distributed or not. If the amounts of normalized gene expression levels were normality distributed, comparison of gene expressions among more than two groups were tested using one-way ANOVA test followed by the Post Hoc tests (Tukey HSD). Otherwise, the Kruskal-Wallis one-way ANOVA test was applied. Pearson and Spearman correlation test were performed to find the potential correlations between different variables. In addition, we used the software REST version 2009 V2.013 to calculate the fold changes of lncRNAs in tumor tissues compared to TANTs. P values < 0.05 were considered statistically significant.

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Results

Demographic, Clinicopathological and reproductive data

The mean age (± standard deviation) of participants in the current study was 45 (± 10.63). Table 2 presents the clinicopathological data of the patients, including tumor size, estrogen receptor (ER) status, progesterone receptor (PR) status, HER-2 status, nuclear grade, histological grade, TNM stage, and lymph node metastasis (LNM). In addition, reproductive characteristics of subjects in the present study were menopausal status, age at menarche, age at first full term pregnancy (FFTP), FTP number, and breastfeeding duration.

Table 2. Clinicopathological and reproductive data of patients

Variables	subgroup	frequency	Valid percent	Cumulative percent
Tumor size	<2.5	18	36	36
	≥2.5	32	64	100
Estrogen receptor	Positive	27	54	54
	Negative	23	46	100
Progesterone receptor	Positive	22	44	44
	Negative	28	56	100
HER-2	Positive	21	42	42
	Negative	29	58	100
Nuclear grade	I	14	28	28
	II	24	48	76
	III	12	24	100
Histological grade	I	9	18	18
	II	27	54	72
	III	14	28	100
TNM stage	I	15	30	30
	II	22	44	74
	III	13	26	100
Lymph node metastasis	Involved	16	32	32
	Free	34	68	100
BMI	<25	16	32	32
	25-29	19	38	70
	≥30	15	30	100
Menopausal Status	Premenopausal	29	58	58
	Postmenopausal	20	40	100
Breastfeeding duration (month)	0	9	18	18
	1-6	18	36	54
	6-24	15	30	84
	>24	8	16	100
Age at Menarche	≤13	24	48	48
	≥14	26	52	100
FTP number	0 (Nulliparity)	8	16	16
	1-2	22	44	60
	≥3	20	40	100
Age at FFTP	<25	24	48	48

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	≥25	26	52	100

Expression of FAL1, HAND2-AS1, and PTCSC3 lncRNAs in tumor and tumor-adjacent normal breast tissue

To determine the relationship of FAL1, HAND2-AS1, and PTCSC3 with the BC malignancy, we firstly performed qRT -PCR assay to measure their expression profiles in 50 breast tumor tissues and 50 tumor -adjacent normal breast tissues (TANTs). As shown in fig1, there was significant difference in the expression levels of FAL1 between breast tumor tissues and tumor -adjacent normal breast tissues. According to these results, the expression of FAL1was evidently higher in breast tumor tissues than that in tumor-adjacent normal breast tissues (P <0.001). Regarding to the lncRNAs HAND2-AS1 and PTCSC3, achieved data indicated significant decreased in breast tumor tissues compared to tumor-adjacent normal breast tissues (P <0.001).

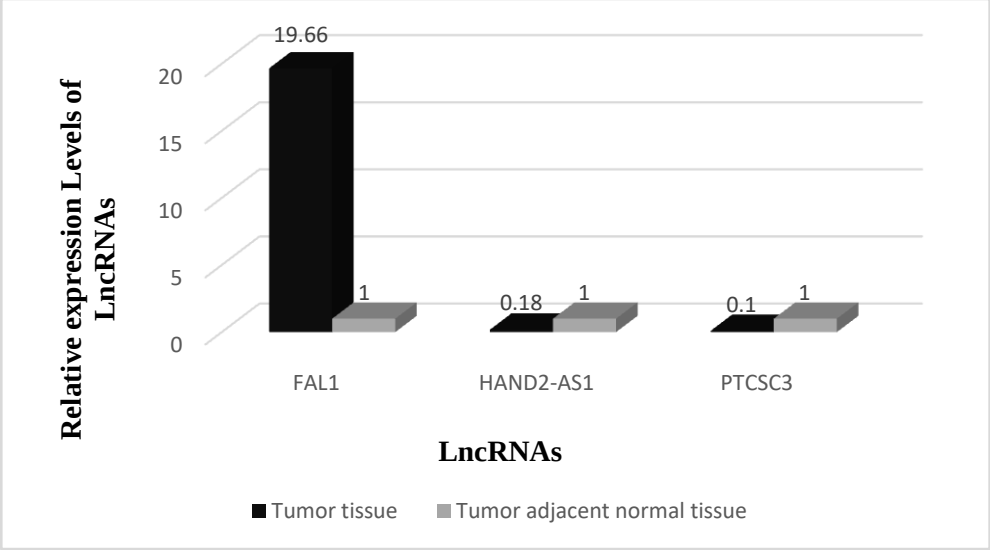

Figure 1. Comparison of lncRNAs FAL1, HAND2-AS1, and PTCSC3 expression levels in tumor tissues and tumor adjacent normal tissues (TANTs). Our results showed significant upregulation of the FAL1 in tumor tissues compared to TANTs (P < 0.001). While, it has been observed that expression levels of HAND2-AS1 and PTCSC3 considerable lower in tumor tissues than TANTs (P values < 0.001).

Expression levels of FAL1, HAND2-AS1, and PTCSC3 in relation to the clinicopathology characteristics of BC patients

ANOVA test was done for comparing the expression levels of three lncRNA genes between the different clinical and pathological subgroups of BC, including tumor size, ER, PR, HER-2, nuclear grade, histological grade, TNM stage, and LNM (Table 3). The achieved data disclosed that there are significant differences in FAL1 expression levels between different subgroups of tumor size, ER, PR, HER2, and nuclear grade (fig 2). The expression levels of FAL1 is higher in tumors with ≥2.5 cm compared to tumors with <2.5 cm (P = 0.003). In addition to, significantly increased level of its expression was observed in ER negative (ER^-) compared to ER positive (ER^+) (P <0.001), PR negative (PR^-) compared to PR positive (PR+) (P = 0.001), and HER-2 negative compared to positive (P <0.001). Besides, a considerable increasing of FAL1 expression level was found in nuclear grades II and III versus grade I (P <0.001). Regarding to lncRNA HAND2-AS1, evaluations showed considerable elevation of its expression levels in in tumors with <2.5 cm compared to tumors with ≥2.5cm (P <0.001), as well as in nuclear grade I compared to

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grades II and III (P <0.001) (fig 3). In relation to the PTCSC3 lncRNA, its expression levels showed notable increasing in patients with $HER2^-$ versus $HER2^+$ (P = 0.012), patients with free lymph node metastasis than those with nodal disease (P <0.001), as well as lower stages of disease (P <0.001) (fig 4). Interestingly, as shown in Table 4 and fig 5, the expression levels of lncRNA FAL1 were significantly promoted in triple negative breast cancer (TNBC) compared to none-TNBC samples.

Table 3. Evaluation of lncRNA expression levels with clinical and pathological variables

lncRNAs		FAL1			HAND2-AS1			PTCSC3		
Variables	Subgroups	Mean	Std. deviation	P-value	Mean	Std. deviation	P-value	Mean	Std. deviation	P
Tumor size (cm)	<2.5	5.78	2.18	**0.003**	12.68	2.26	**<0.001**	12.38	3.14	0.155
	≥2.5	7.65	1.36		9.07	1.92		11.17	2.67	
ER	Positive	6.08	1.26	**<0.001**	9.96	2.63	0.241	11.48	2.86	0.737
	Negative	8.03	2.03		10.85	2.71		11.76	2.95	
PR	Positive	6.03	1.40	**0.001**	10.41	2.99	0.917	11.15	2.98	0.327
	Negative	7.72	1.95		10.33	2.46		11.97	2.80	
HER2	Positive	5.76	1.51	**<0.001**	10.95	2.52	0.195	10.40	2.94	**0.012**
	Negative	7.86	1.68		9.95	2.75		12.48	2.53	
LNM	Involved	7.27	1.57	0.420	9.69	2.12	0.193	8.69	1.94	**<0.001**
	Free	6.84	2.06		10.69	2.87		12.98	2.12	
Nuclear grade	1	5.31	2.01	**<0.001**	13.47	1.86	**<0.001**	12.03	3.17	0.812
	2	7.69	1.52		10.07	0.92		11.41	3.01	
	3	7.49	1.31		7.34	1.91		11.50	2.39	
Histological grade	1	6.65	2.56	0.196	12.19	3.15	0.62	13.19	2.93	0.194
	2	6.68	1.85		10.15	2.47		11.25	2.73	
	3	7.76	1.40		9.61	2.39		11.30	2.99	
TNM stage	1	6.89	2.24	0.707	10.93	2.95	0.339	14.72	1.05	**<0.001**
	2	6.81	1.82		10.51	2.67		11.52	1.67	
	3	7.36	1.74		9.47	2.30		8.16	1.60	
P-values less than 5% are considered significant and shown in bolded forms.										

Table 4. Relative expression levels of studied lncRNAs in TNBC and none- TNBC samples

lncRNAs	TNBC status	Mean	Std. Deviation	P
FAL1	Positive	8.80	1.27	**<0.001**
	Negative	5.95	1.37	
HAND2-AS1	Positive	10.41	2.44	0.933
	Negative	10.35	2.85	
PTCSC3	Positive	12.22	2.75	0.257
	Negative	11.26	2.93	
P-values less than 5% are considered significant and shown in bolded forms.				

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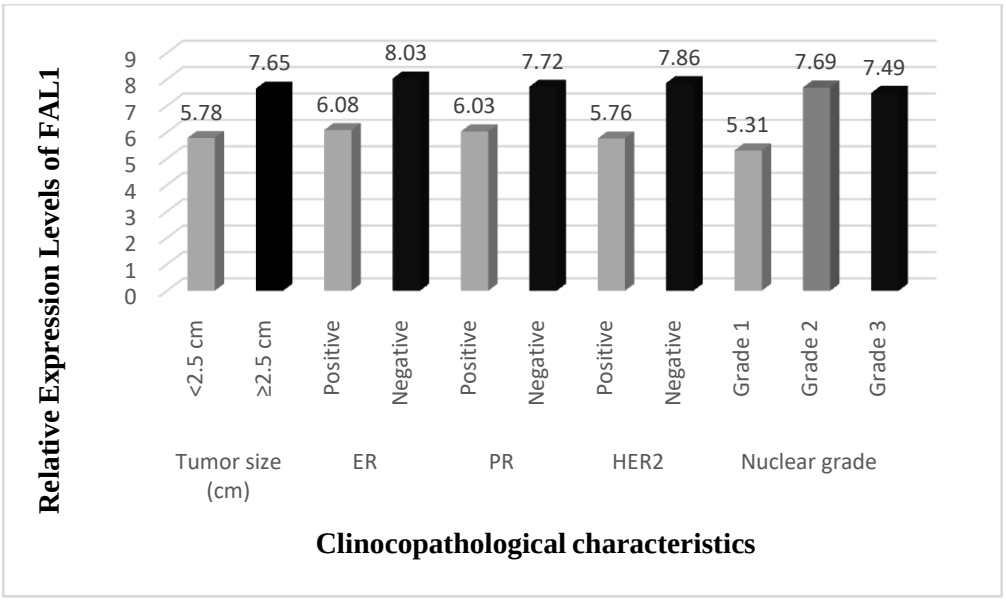

Figure 2. Significant differences in expression levels of the FAL1 in subgroups of some BC clinicopathological data. Considerable elevation in expression levels of the FAL1 was found in larger tumor sizes ($P = 0.003$), negative status of receptors ER ($P < 0.001$), PR ($P = 0.001$), HER2 ($P < 0.001$), as well as grade II and III versus grade I ($P < 0.001$).

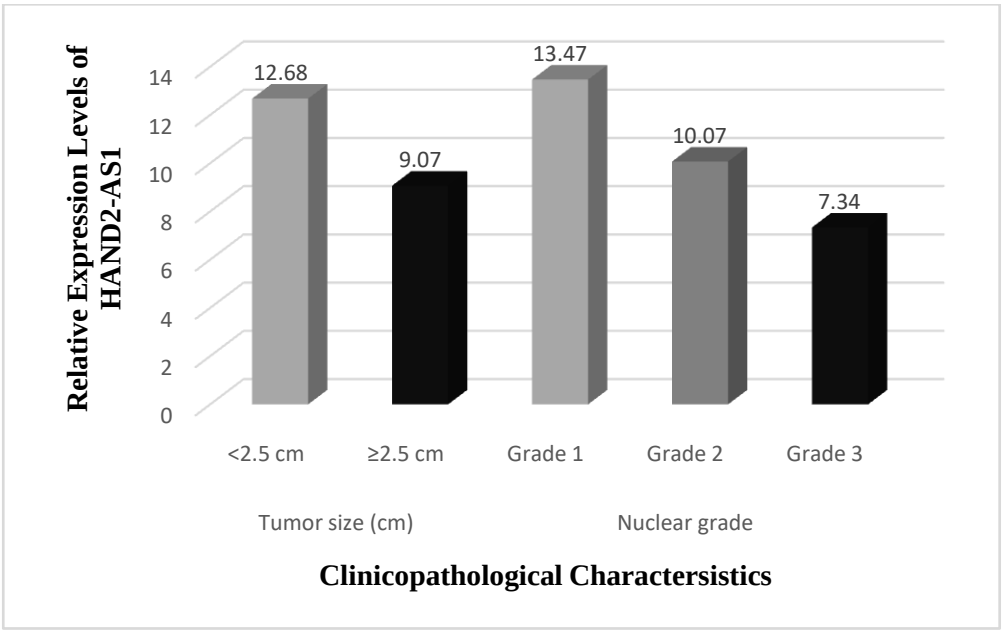

Figure 3. Significant differences in expression levels of the HAND2-AS1 in subgroups of some BC clinicopathological data. Expression levels of lncRNA HAND2-AS1 significantly higher in smaller tumor sizes and early grades of BC tumors (P values < 0.001).

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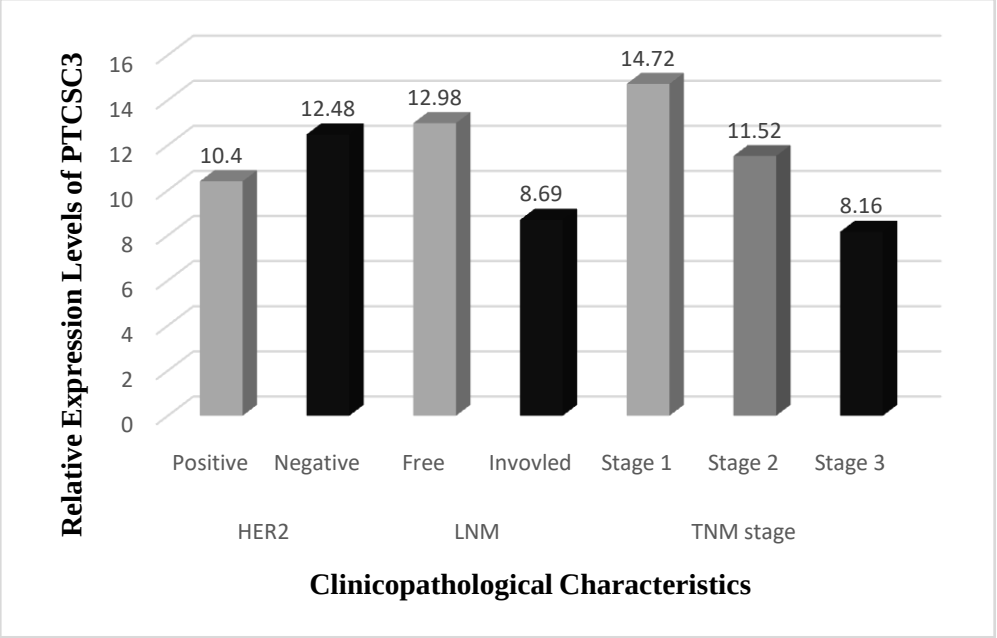

Figure 4. Significant differences in expression levels of the PTCSC3 in subgroups of some BC clinicopathological data. Our findings showed notable elevation of lncRNA PTCSC3 expression levels in BC patients with negative status of HER2 ($HER2^-$) ($P = 0.012$), free- lymph node metastasis (LNM) ($P < 0.001$), as well as early stages of disease ($P < 0.001$).

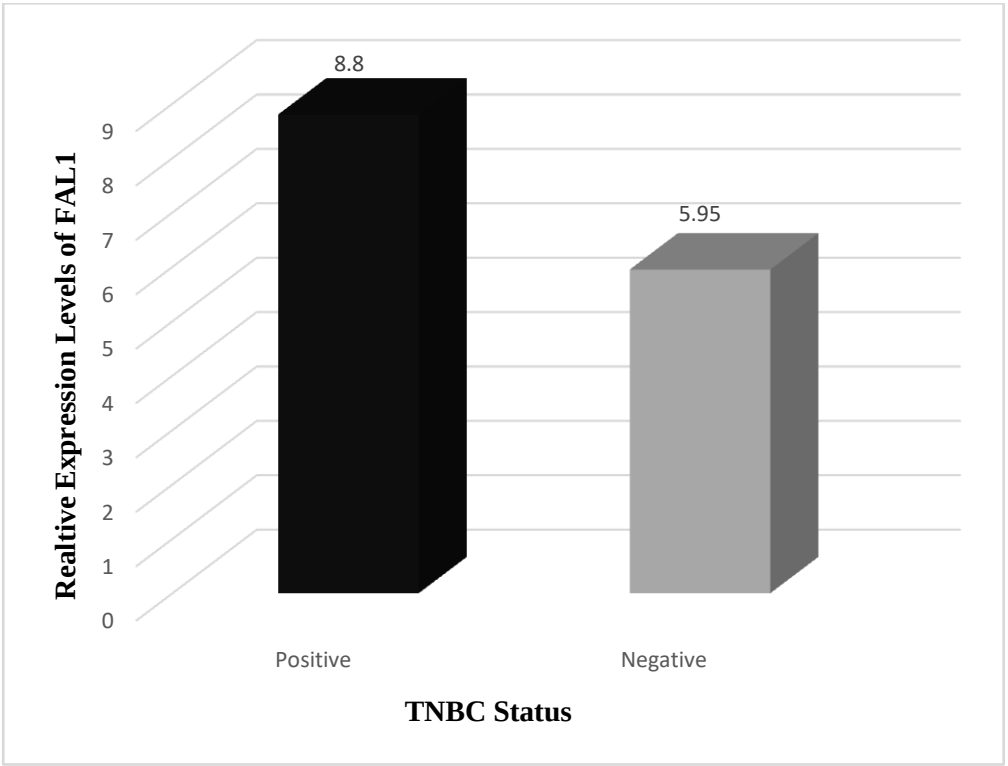

Figure 5. Expression levels of lncRNA FAL1 in triple- negative breast cancer (TNBC) samples versus none-TNBC samples. It has been observed clearly that expression levels of the FAL1 higher in TNBC samples compared to none- TNBC samples ($P < 0.001$).

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Expression analysis of FAL1, HAND2-AS1 and PTCSC3 in demographic and reproductive characteristics

Expression levels of lncRNAs FAL1, HAND2-AS1 and PTCSC3 in demographic and reproductive variables of patients were analyzed by independent T- test, one-way ANOVA, as well as Pearson correlation (Table 5). Interestingly, we showed significant differences in expression levels of FAL1 and HAND2-AS1 in subgroups of BMI index. As shown in table 5, we showed considerable reduction in expression levels of FAL1 in patients with BMI <25 than those with BMI > 25 (P = 0.012). Inversely, significant increasing in expression of HAND2-AS1 was observed in patients with lower BMI index (P = 0.001). In addition, our analyses showed notable differences in expression levels of FAL1 and HAND2-AS1 in patients with different rates of lactation (P < 0.001 and P = 0.019, respectively). Patients with higher breastfeeding duration showed lower expression of the FAL1. On the contrary, with increasing lactation, HAND2-AS1 was elevated. Furthermore, we observed higher expression levels of lncRNA FAL1 in nulliparous patients compared to patients who had 1-2 children (P = 0.001). Finally, our analyses showed significant differences of the HAND2-AS1 expression between subgroups of menopausal status (P = 0.048). We found that HAND2-AS1 expression rates significantly increased in postmenopausal women compared to premenopausal women.

Table 5. Evaluation of lncRNA expression levels in subgroups of reproductive characteristics

lncRNAs		FAL1			HAND2-AS1			PTCSC3		
Characteristics	Subgroups	Mean	Std. deviation	P-value	Mean	Std. deviation	P-value	Mean	Std. deviation	P
Menopausal Status	Premenopausal	7.04	1.71	0.649	9.78	2.64	**0.048**	11.32	2.62	0.500
	Postmenopausal	6.78	2.18		11.31	2.56		11.89	3.26	
Age at Menarche	≤13	7.23	2.03	0.376	10.88	2.35	0.198	12.16	2.75	0.196
	≥14	6.74	1.80		9.90	2.91		11.10	2.95	
Age at FFTP	<25	7.28	2.06	0.292	10.64	2.60	0.504	12.26	3.02	0.128
	≥25	6.70	1.76		10.12	2.77		11.01	2.66	
BMI	<25	5.92	1.93	**0.012**	12.13	1.97	**0.002**	10.85	3.86	0.147
	25-29	7.16	1.73		10.02	2.35		12.62	2.02	
	≥30	7.88	1.64		8.92	2.79		11.14	2.34	
Breastfeeding duration (month)	0	9.17	1.51	**< 0.001**	10.25	2.82	**0.019**	10.80	2.51	0.172
	1-6	7.20	1.25		9.37	2.62		10.87	2.42	
	6-24	6.81	1.46		10.31	2.07		12.09	3.58	
	>24	4.31	0.63		12.86	2.48		13.27	2.20	
FTP number	0 (Nulliparity)	9.13	1.61	**0.001**	10.55	2.86	0.973	10.91	2.66	0.248
	1-2	6.74	1.65		10.29	2.15		11.10	3.16	
	≥3	8.38	1.75		10.39	3.21		12.44	2.54	

P-values less than 5% are considered significant and shown in bolded forms.

Discussion

BC with 268,600 estimated new cases and 41,760 estimated deaths in the United States in 2019 is the most common malignancy in women. It accounts for 30% of all new cancer diagnoses in women and also ranks the second in the cancer death of female (Siegel and others 2019). Early detection and improvement in standard care have increased survival of patients. However, due to the complex nature, heterogeneity of a single tumor, multiple disease subtypes, and high recurrence rate, BC cannot be completely cured (Jiang and others 2018). In order to achieve the

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desired results in the treatment of BC, we need to know more about genetic factors and mechanisms involved in this malignancy. LncRNAs are a class of ncRNA transcripts >200 nucleotides in length and have key roles in various types of cellular mechanisms and malignancies including BC (Abdollahzadeh and others 2019; Bartonicek and others 2016; Soudyab and others 2016; Yarmishyn and Kurochkin 2015) . The functionality of lncRNAs FAL1, HAND2‑AS1, PTCSC3 has been characterized in many types of cancers; however, their roles in BC is currently unknown.

Our results showed significant increasing in expression levels of FAL1 lncRNA in tumor tissues versus paired tumor adjacent tissues. Furthermore, the result of the current study revealed that there are considerable differences in the expression rate of these lncRNAs between clinical and pathological subgroups of BC including tumor size, ER, PR, HER2, and nuclear grade.

Notably, expression levels of lncRNAs FAL1 was upregulated in tumors with higher sizes and nuclear grades. These results suggested that lncRNA FAL1 may has oncogenic role in BC which is consistent with other studies (Li and others 2018; Liang and others 2018; Pan and others 2017; Wang and others 2019a; Wu and others 2018; Yang and others 2019; Zhu and others 2018). Previous studies revealed that FAL1 plays outstanding oncogenic activity in various types of cancers through different targets. For instance, it is demonstrated that lncRNA FAL1 can promote thyroid cancer via E2F transcription factors (Jeong and others 2016). In addition, it is showed that FAL1 may promote oncogenesis and progression of different cancers through the PTEN/AKT signaling axis (Liang and others 2018; Pan and others 2017; Zhu and others 2018). Other targets of FAL1 including mitochondrial fission protein dynamin-related protein 1 (DRP1) in Esophageal cancer (Yang and others 2019), miR-637/NUPR1in CRC (Wang and others 2019a).

Moreover, the results of our study disclosed significant elevation of FAL1 expression in negative status of receptors ER, PR, and HER2 versus their positive status. ER, as a critical predictive omarker, activated via binding estrogen and subsequently up- or down- regulate target genes that control endocrine response and cell cycle progression (Doisneau-Sixou and others 2003; Shang and others 2000). Tamoxifen, as a competitive antagonist of ER, has been the mainstay therapy for ER^+ BC that accounts for at least 70% of all BCs (Musgrove and Sutherland 2009). Despite great success in reducing mortality and improving survival of BC patients, tamoxifen resistance (TamR) is frequently led to the onset of resistance and is a major cause of BC recurrence and mortality (Clarke and others 2001; Ring and Dowsett 2004). Thus, it has become indispensable to understand the mechanisms of TamR acquisition and improve treatment for BC. PR as one of the ER-regulated genes and a prognostic marker performs important roles in lobuloalveolar development of the gland mammary, BC, as well as responding to selective ER modulator (SERM) therapies (Cui and others 2005). The approximately 15% of BC patients who have HER2 overexpressing and/or amplified tumors have a good response to treatment with combination of trastuzumab (Herceptin) and adjuvant chemotherapy (Slamon and others 2001). However, the majority of patients with metastatic BC demonstrate disease progression within 1 year of Herceptin treatment initiation. Many studies identified several molecular mechanisms for development of trastuzumab resistance (Nahta and others 2006).Interestingly, it is showed that the expression levels of lncRNA FAL1 was notably higher in patients with TNBC than those with none-TNBC tumors. Patients with TNBC subgroup, which consists of approximately 12 to 17% of BC women, are defined as patients which their tumors lack expression of ER, PR, and HER2. These patients have a relatively poor outcome and cannot be treated with therapies targeted to ER and HER2 (Foulkes and others 2010). LncRNAs, as an amazing area in the post-genome period, have been discovered to play important roles in the initiation and development of the TNBC, as well as considered as diagnostic biomarkers and therapeutic targets (Lv and others 2016).Accordingly, we suggested that lncRNA FAL1 may play crucial roles in pathogenesis and clinical outcome of TNBC.

The functional role of HAND2-AS1 in BC is unknown. Therefore, we examined expression levels of HAND2-AS1 in BC and characterized its clinical significance in BC progression. In the present study, we found that the expression levels of HAND2-AS1 lncRNA significantly decreased in tumor tissues compared to TANTs. Significant

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 3621 - 3636
Received 25 April 2021; Accepted 08 May 2021.

reduction in expression levels of HAND2-AS1 in tumor tissues compared with TANTs has been observed in several types of cancers (Chen and others 2019a; Chen and others 2019b; Kang and others 2018; Liu and others 2019; Miao and others 2019; Wang and others 2019b; Yang and others 2018; Zhou and others 2018).In addition, our results showed that lower expression of the HAND2-AS1 was associated with worse outcomes in BC including larger tumor sizes and higher nuclear grades. Recently, studies reported that down-regulation of lncRNA HAND2-AS1 is correlated with poor prognosis in different cancers, such as higher tumor grades, advanced stages, metastasis, and higher recurrence rates in EEC and CRC patients (Yang and others 2018; Zhou and others 2018), as well as TNM stage in melanoma (Liu and others 2019). Furthermore, other studies suggested that lncRNA HAND2-AS1 can be used as a diagnostic biomarker in early stages of BC through evaluation of its expression levels in plasma (Miao and others 2019). Our findings suggested a potential tumor suppressive role for lncRNA HAND2-AS1 in BC progression. However, functional analysis is required to confirm its role in tumorigenesis of BC. Besides, recently studies revealed that HAND2-AS1 exerts its anti-oncogenic effects through different pathways in other malignancies, such as neuromedin U (NMU) in EEC (Yang and others 2018), HIF1α and GLUT1-mediated energy metabolism in OS(Chen and others 2019b; Kang and others 2018), BMP signaling in HCC (Wang and others 2019b), miR-1275/KLF14 axis in CRC (Zhou and others 2018), transforming growth factor β (TGF-β1) in NSCLC (Miao and others 2019), Rho-associated protein kinase 1 (ROCK1) in melanoma (Liu and others 2019), miR-340-5p/ BCL2L11 axis in ovarian cancer (Chen and others 2019a), and miR-21 in ESCC (Miao and others 2019).

In the present study we showed that expression levels of lncRNA PTCSC3 significantly decreased in breast tumor tissues versus TANTs which is consistent with findings of previous studies in many types of cancers including thyroid cancer (Fan and others 2013; Wang and others 2018; Wang and others 2017b), glioma (Wang and others 2017b), TNBC (Xiao and others 2019), and LSCC (Xiao and others 2019). Moreover, our results revealed that upregulation of PTCSC3 correlated with some clinicopathological variables including $HER2^-$ status, LNM, as well as early stages of BC. Similarly, Wang et al. study reported slight decreases of PTCSC3 expression levels in early stages of TNBC compared to higher stages, while the changes were not significant (Xiao and others 2019). According to these Wang et al. results, we suggested that lncRNA PTCSC3 has an anti-tumorigenic effect in BC. However, the potential pathogenic mechanism was still unclear in BC and required more investigations. Previous studies illustrated that PTCSC3 play its anti-tumorigenic role through down-regulation of different targets/ or pathways, such as Wnt/β-catenin signaling pathway (Wang and others 2017b), lncRNA H19 (Xiao and others 2019), and lncRNA HOTAIR (Xiao and others 2019). In addition, Xiao et al. demonstrated that lncRNA PTCSC3 can be a useful biomarker in diagnosis of LSCC via evaluating of its expression levels in plasma (Xiao and others 2019).

Interestingly, our study deciphered significantly increased of FAL1 and decreased of HAND2-AS1 in BC patients with never breastfeeding women compared to women who have had breastfeeding durations. Many previous literatures have illustrated important effects of breastfeeding duration in reducing the risk of BC (Anothaisintawee and others 2013; lancet 2002; Victora and others 2016). In addition, the results of the current study showed significant increase of lncRNA FAL1 in nulliparous patients than parous women. Previously, the importance of parity role in reducing the risk of BC has also been confirmed (lancet 2002). Another reproductive characteristic that display significant differences in expression levels of the HAND2-AS1 between its subgroups was menopausal status. Our data showed that HAND2-AS1 expression rate significantly increased in postmenopausal women versus premenopausal women. The results of previous literatures linking reproductive characteristics to reduced BC risk is controversial which can be due to the sever heterogeneity of BC (Ambrosone and others 2014; Islami and others 2015; Lambertini and others 2016; Millikan and others 2008; Palmer and others 2014; Shinde and others 2010). Furthermore, we found that expression levels of the FAL1 considerably increased in patients with higher BMI index. While, expression levels of lncRNA HAND2-AS1 showed negative correlation with BMI index in BC patients. A lot of studies previously reported that overweight and obesity might be associated with risk and poorer prognosis of BC (Liu and others 2018; Sun and others 2018). Based on present study, we suggest that BMI index as well as reproductive characteristic such as menopausal status, lactation, and number of pregnancies may influence the

expression levels of lncRNAs FAL1 and HAND2-AS1 and subsequently alter the susceptibility of BC risk. However, further studies are required to confirm the results of our work.

In summary, we found upregulation of lncRNA FAL1 and downregulation of lncRNAs HAND2-AS1 and PTCSC3 in BC tumor tissues compared to TANTs. Furthermore, our data revealed that dysregulation of lncRNAs FAL1, HAND2-AS1, and PTCSC3 correlates with worse outcomes of disease. Finally, we observed significant differences in expression levels of FAL1 and HAND2-AS1 in subgroups of some characteristics, including BMI index, menopausal status, breastfeeding duration, and FTP number. Therefore, we suggest lncRNAs FAL1, HAND2-AS1, and PTCSC3 as novel lncRNAs involved in pathogenesis of BC, which can be used as important biomarkers in prognosis and therapeutic targets of BC.

Acknowledgments

The authors would like to thank staffs at Imam Khomeini Hospital for their contributions to breast tissue sampling and data collection as well as the patients who took part in this study.

Author Disclosure Statement

No competing financial interests exist.

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