Circulating long non coding RNA H19 as a diagnostic biomarker of breast cancer

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Abstract

Background

LncRNA H19 is a carcinogenic gene located at 11p15.5 of human chromosome, which is abnormally expressed in some types of tumors and acts as a tumor suppressor gene (TSG). Early diagnosis of breast cancer is crucial for patient treatment and prognosis. Long non coding RNA have potential roles in tumor initiation and differentiation.

Aim: the objective of this study was to investigate whether circulating LncRNA H19 could be used as a biomarker in diagnosis of breast cancer. Besides we aimed at evaluating the association of LncRNA H19 with TNM staging in order to predict cancer prognosis.

Methods: This prospective case-control study was carried out in Clinical Pathology Department and General Surgery Department, Faculty of Medicine-Zagazig University. The study included eighteen apparently healthy females attending outpatient laboratory (Group A) and 36 adult female patients, were classified into 2 groups: Group B included eighteen adult female patients newly diagnosed with breast cancer, admitted to General Surgery Department diagnosed by both mammography and histological examination of biopsy samples and Group C, included eighteen adult female patients with benign breast lesions. LncRNA H19 was assessed in plasma using quantitative real-time polymerase chain reaction (RT-PCR) on applied bio system.

Results: circulating long non coding RNA H-19 was significantly higher among studied BC cases than healthy controls or cases with benign breast lesions. A significant increase was found in expression level of circulating H-19 among BC cases with TNM stages III/IV, tumor grades II and III and also those who had positive ER\PR expression. The expression level of long non coding RNA H-19 were used to discriminate breast cancer patients from controls and benign breast lesions with an AUC 0.931 with sensitivity 83.3% and specificity 86.1%. However CA15-3 had a sensitivity and specificity (77.8% and 80.7% respectively). Moreover, long non coding RNA H19 showed better performance in differentiation between TNM stages (I/II) and (III/IV) with sensitivity of 74.3% and specificity 82.8% versus CA15-3 sensitivity 71.4% and specificity 75.9% .

Conclusion: Plasma H19 expression levels were up regulated in patients with BC. Moreover, long non coding RNA H19 showed better performance than CA15-3 in diagnosis and prognosis of breast cancer differentiating early stages I/II from late stages III/IV. Thus it could be used as promising biomarker in diagnosis and prognosis of breast cancer.

Keywords: long non coding RNA H19, Breast cancer

INTRODUCTION

Breast cancer is the most common worldwide cancer for women. Referring to World Health Organization (WHO), about 1.7 million new cases of cancer breast were diagnosed yearly and about 500 thousands women with breast cancer died annually **(1).**

Providing the best chance for cure, the diagnosis must be at early stage. Screening tests can reduce rate of mortality **(2).**

Though, traditional screening and subsequent management of BC patients are based on serological biomarkers, including CA15-3 and CEA **(3).**

Long non-coding RNAs (LncRNAs) are RNAs with more than 200 nucleotides and are mostly transcribed by RNA polymerase II from different regions across the genome. They are now recognized as key regulators of cellular function through altered mechanisms such as epigenetic and non-epigenetic mechanisms under both normal and pathologic disorders, with cancer **(4)**

Furthermore, noncoding RNAs are found stable in human body liquid and their expression levels may act as clinical indicators **(5).**

lncRNAs have been identified exhibiting oncogenic roles in breast cancer, including H19, HOTAIR, MALAT-1, CCAT1, CCAT2 and UCA1 **(6).**

LncRNA H19 gene is maternally expressed and coded a long noncoding RNA that has been reported previously involved in the tumor progression **(7).**
LncRNA H19 is a carcinogenic gene located at 11p15.5 of human chromosome, which is abnormally expressed in some types of tumors and acts as a tumor suppressor gene (TSG). According to the evidence, it suggests that genetic changes in lncRNA H19 play an important role in cancer development **(8).**
This study aimed at investigating circulating LncRNA H19 as a biomarker in diagnosis of breast cancer. Besides evaluating the association of LncRNA H19 with TNM staging in order to predict the cancer prognosis

METHODS

This prospective case-control study was carried out in Clinical Pathology Department and General Surgery Department, Faculty of Medicine-Zagazig University. Approval for the study was obtained from International Review Board (IRB), Faculty of Medicine-Zagazig University. Sample collection started in April 2022, and ended in October 2022. The study included eighteen healthy control female subjects (group A) without any other malignant tumours and 36 adult female patients including eighteen female patients were newly diagnosed with breast cancer by histological examination of biopsy samples (group B) and eighteen female patients with benign breast lesions (group C). Written informed consent forms were obtained from all participants before sample collection for permission to use their samples and clinical data in this study according to the Declaration of Helsinki. Blood samples were collected before surgical treatment, chemotherapy, or radiotherapy .Tumor staging was determined through tumor, node, metastasis (TNM) staging according to The American Joint Committee on Cancer (AJCC).

Plasma samples

Plasma samples were collected in ethylenediaminetetraacetic acid (EDTA) containing tubes; EDTA tubes were separated according to two-step centrifugation protocol (first centrifuge at 2,000*g* for 5 Min then the second at 12,000*g* for 5 Min both done at 4 °C) to completely remove the cellular sediments, then samples were aliquoted into two tubes; one used to assess CA15-3 values immediately, and second tube was stored at −80 °C until total RNA extraction.

Measuring of Ca15-3 levels

Plasma CA15-3 levels were measured using cobas e 602 analyzer (Roche Diagnostics, Mannheim, Germany) by electrochemiluminescence immunoassay.

Extraction of RNA and real-time PCR

RNA was isolated from 2 mL of supernatant plasma using "(Genaid, Taiwan).The kit combines phenol, chloroform and guanidine isothiocyanate based scalable solution for extracting high-quality total RNA according to the manufacturer's instructions. RNA concentration and purity were measured by spectrometer UNICO UV2000. The complementary DNA had been reversed transcribed from the isolated purified RNA using TOPscript™ RT DryMIX(dT18/dN6 plus) is formulated for maximal stability of TOPscript™ Reverse Transcriptase that is dried with reaction buffer, dNTP mixture, stabilizer, oligo dT primer and random hexamer primer. This product is ready to use upon the addition of template RNA in distilled water to each tube (Enzynomics, Korea).

For quantitative real-time PCR (qRT-PCR) reaction,

The LncRNA (H19) was amplified with qPCR SYBR® Green. PCR Master Mix (TOPreal™ qPCR 2X PreMIX(SYBR Green with low ROX) (Enzynomics, Korea). The fluorescent dye in the master mix intercalates into the amplification product during the PCR process and enables the rapid analysis of target DNA. The Master mix contains all reagents required for qPCR (except template and primer) in a ready-to-use solution

20 µL of the reaction mixture was used containing TOPreal™ qPCR 2X PreMIX(SYBR Green with low ROX) 10 µl ,Template DNA 1 µl , (5~10 pmol/µl) 1 µl of each primer and complete with sterile water (RNase free) up to 20 µl

The amplification was performed using (Applied Biosystem,Singapore)RT-PCR System. The RT-PCR amplification mix was done using the following cycling conditions, initial activation step: 15 min at 95°c to activate HotStar Taq DNA Polymerase then 40 cycles of denaturation at 94 ℃ for 10 seconds, annealing at 60 ℃ for 15 seconds and extension at 72 ℃ for 30 seconds

A melting curve analysis was prepared to confirm the specificity of the PCR products. For the melting "dissociation" curve one cycle was done started with 95 º c for 1 minute then 55 º c for 30 seconds followed by 95 ºc for 30 seconds. GAPDH was used as the housekeeping gene. The gene-specific primer pair sequences for H19 were; H19 forward: 5 ATCGGTGCCTCAGCGTTCGG-3′ ′; H19 reverse: 5′- CTGTCCTCGCCGTCACACCG-3″, and for GAPDH were; GAPDH forward, 5′-CACCAGGGCTGCTTTTAACTC -3′; GAPDH reverse, 5′- GACAAGCTTCCCGTTCTCAG-3′ **(9)**. The relative expressions of lncRNA were calculated using the $2^{-\Delta\Delta CT}$ method.

Data Acquisition and Processing

Using the software of the thermal cycler, the following steps were done: Visual check of amplification curves for homogeneity. Determination of baseline fluorescence values. Setting the threshold fluorescence. Visual check of dissociation curves for non-specific fluorescence signals

Next, threshold cycle (CT) values were registered for each sample well, and were normalized against RNU-6. Fold changes of long non coding RNA expression were calculated using $2^{-\Delta\Delta CT}$ according to **Livak and Schmittgen (10)** where:

Fold Change = $2^{-\Delta\Delta CT}$

ΔΔCt = ΔCt (patients**) – ΔCt (control)**

ΔCT (relative expression) = CT lncRNA of interest – CT housekeeping gene

Statistical Analysis

Data were analyzed using IBM SPSS 23.0 for windows (SPSS Inc., Chicago, IL, USA) and NCSS 11 for windows (NCSS LCC., Kaysville, UT, USA). Quantitative data were expressed as mean ± standard deviation (SD). Qualitative data were expressed as frequency and percentage. The following

tests were done: Independent sample t test, Mann-Whitney test for not normally distributed data, chi-square and fisher exact for analysis of qualitative data, ROC curve analysis for validity data. Probability (P-value): P-value <0.05 was considered significant, P-value <0.001 was considered as highly significant and P-value >0.05 was considered insignificant.

RESULTS:

Table (1) General characteristic features of all participants

	Studied groups			F test\ X^{2*}	P
	Group A	Group B	Group C		
Age	43.3 ± 7.6	49.4 ± 13.1	45.4 ± 7.9	1.82	0.18 NS
BMI	27.8 ± 2.94	29.1 ± 2.95	26.9± 3.35	2.26	0.115 NS
Menarche age	12.3 ± 0.89	12.1 ± 0.76	12.2 0.99	0.166	0.848 NS
Marital state					
Single	5 (27.8%)	4 (22.2%)	7 (38.9%)	1.24*	0.537
Married	13 (72.2%)	14 (77.8%)	11 (61.1%)		NS
n. of parity					
No	5 (27.8%)	5 (27.8%)	8 (44.4%)		
1-2	8 (44.4%)	9 (50.0%)	7 (38.9%)	1.16	0.674
3-4	5 (27.8%)	4 (22.2%)	3 (16.7%)		NS

No significant difference was found among studied groups regarding age, sex, marital state or number of parity.

Table (2 Expression of H19 in studied groups:

	Studied groups			KW	P
	Group A	Group B	Group C		
H19 Median (Range)	0.98 (0.96-1)	10 (1-23)	3 (1-5)	4.82	<0.001 HS

The results revealed that the expression of circulating H19 were significantly higher among studied BC cases than healthy controls or cases with benign breast lesions.

Table (3): Expression levels of H19 in breast cancer patients in relation to pathological features.

	H19	
	Median (Range)	P*
TNM stage		
I-II	7 (1-14)	**0.02**
III-IV	12 (10-23)	**S**
Grade		
I	7 (1-14)	**0.02**
II- III	12 (10-23)	**S**
ER\ PR		
Negative	2 (1-5)	**0.02**
Positive	10 (3-23)	**S**
HER 2		
Negative	11 (1-23)	0.33
Positive	8 (3-12)	NS

*Mann-Whitney test of non-parametric data

There was statistical significant increase in expression of circulating H19 among BC cases with TNM stages III/IV, tumor grades II and III and also those who had positive ER\PR expression.

Table (4) Reliability data of investigated parameters for differentiation of breast cancer patients and controls

	AUC	Sensitivity	Specificity	PPV	NPV	Accuracy	P
H19	0.931	83.3%	86.1%	71.4%	91.2%	85.2%	<0.001
CA 15-3	0.862	77.8%	80.7%	66.7%	87.9%	79.6%	<0.001
H19+ CA15-3 (Combined)	0.894	88.9%	85.1%	76.2%	93.9%	87.1%	<0.001

Table (5) Reliability data of H-19 & CA15-3 for differentiation between stages (I/II) and (III/IV) among BC cases.

	AUC	Sensitivity	Specificity	PPV	NPV	Accuracy	P
H19	0.831	74.3%	82.8%	71.4%	81.8%	77.8%	0.02
CA15-3	0.799	71.4%	75.9%	62.5%	80.0%	72.2%	0.04

Difference between areas = 0.06, Z = -0.521, p-value=0.211 (NS), AUC, area under curve; ROC curve: Receiver Operating Characteristic curve, PPV: Positive Predictive Value, NPV: Negative Predictive Value. AUC: Area Under curve. P < 0.001 is high significant.

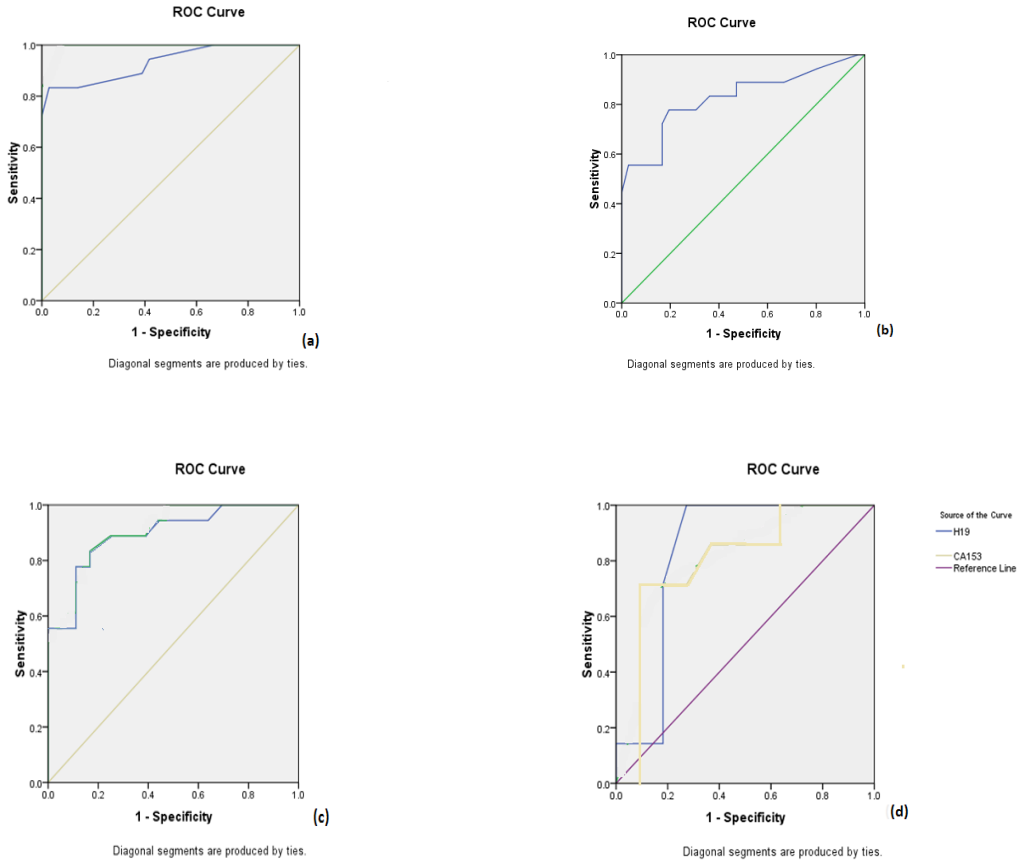

Figure (1) Receiver operating characteristics (ROC) curve

(a) H19 as predictors of BC cases,(b) CA15-3 as predictors of BC cases,(c) H19 combined with CA 15-3 as predictors of BC cases,(d) H19and CA15-3 to discriminate between TNM stages (I/II) and (III/IV) among BC cases.

By using Roc curve, H19 had an AUC 0.931 with sensitivity 83.3% and specificity 86.1% when discriminating patients of breast cancer from healthy subjects and patients with benign lesions. While comparing the diagnostic value of the lncRNAs H19 to CA15-3, the results revealed that H19 had a higher diagnostic value than CA15-3 that have AUC (0.862), sensitivity and specificity 77.8% and 80.7% respectively. In addition, H19 showed higher sensitivity 74.3% and specificity 82.8% with AUC 0.831 than CA15-3 with sensitivity 71.4% and specificity 75.9% in differentiation between TNM stages (I/II) and (III/IV)

DISCUSSION

In worldwide, the most common malignancy and the first leading cause of cancer death among women is breast cancer **(11).** Basically, early diagnosis of breast cancer is the key to improve the patient's survival rate **(12).**

The sensitivity and specificity of traditional markers such as CA15-3 in breast cancer diagnosis is insufficient, particularly in early stages **(13).**Therefore, it is essential to develop noninvasive markers for diagnosis of the early stage of breast cancer. Numerous studies have indicated that circulating nucleic acids are detectable in human peripheral circulation system **(14).**

A study demonstrated that LncRNA H19 was highly expressed in breast cancer cells, and they were related to the occurrence of tumor induced inflammatory response **(11).**

In this work, the analysis of the expression level of LncRNA H19 and its relation with clinical and pathological findings were done. Moreover, ROC curves analysis were established and analyzed to show the diagnostic potentials of this biomarker.

In the current study, the general characteristics of studied groups showed that there was no significant difference between groups as all participants were females ,the average age of breast cancer patients was 49 years, benign cases and controls were of similar age distribution as BC patients,(p=0.18). The body mass index (BMI) and menarche were considered as risk factors for BC; thus, the comparison of BMI and menarche age between BC group , control group and benign group ,the results showed no statistically significant difference (p=0.115;p=0.848).

These results were in agreement with **Xu N et al (15)** and **Olaogun et al (16)** as the average age for their patients with breast cancer was 51years and 49 years for healthy people, also no difference in BMI and menarche age (p=0.4;p=0.3).

The current study reported that, plasma H19 was significantly up-regulated in patients with BC compared to those with benign breast lesions and control groups. (p=<0.001).

These results went with **Alipoor et al (17),** and **Pourramezan et al (18),** who reported that there was higher expression level of H19 in breast cancer patients when compared to control.

Also **Zhong et al (19)** showed significant increase in the expression levels of H19 in breast cancer patients when compared to benign breast diseases and healthy subjects (P< 0.001), as H19 plays differential roles depending on the tissue type and developmental stage **(8).**

H19 is beneficial in the development of BC, probably by one the following mechanisms. 1st, the H19 derived miR-675 gives rise to two functional microRNAs, miR-675-5p and miR-675-3p **(20). Vennin et al** had identified that the overexpression of miR-675-5p in BC cell lines induces the downregulation of c-Cbl and Cbl-b proteins, and promotes the proliferation and metastasis of tumor cells **(21). Matouk et al** found

that H19 upregulates Slug expression, resulting in the suppression of E-cadherin through mir-675 to regulate epithelial-mesenchymal transition of BC and promote invasion and metastasis of BC cells **(22).** 2nd, **Li et al** revealed that H19 upregulates DNA methyltransferase DNMT1 by sponging miR-152, thereby promoting BC cell proliferation and invasion **(23).** 3rd, but not the least, **Barsyte-Lovejoy et al** demonstrated H19 as a Myc-up-regulated gene that potentiates the tumorigenic phenotype of BC cells **(24).**

The expression levels of H19 were related with pathological features of patients with breast cancer as TNM stages III/IV (p=0.02), tumor grade II/III (p=0.02) and ER/PR positive receptor expression (p=0.02) but not correlated with Her-2/ neu expression level. These results went with **Zhong et al (19)** who revealed a correlation between H19 expression levels and pathological features such as TNM stages, and the presence of estrogen and progesterone receptors; however they found significant correlation of H19 with (Her2/neu) expression level. C-erbB-2(Her-2/neu) is a member of the epidermal growth factor family, which has been reported, overexpressed in BC, and the overexpression of c-erbB 2 was correlated with a poor prognosis.

In addition, these results agreed with **Zhang et al (6)** as regard correlation between H19 and positive expression of ER/PR receptor, nevertheless they found significant correlation with C-erbB-2(Her-2/neu).

On the other hand, **Pourramezan et al (18)** found no significant difference regarding TNM staging and ER/PR receptor expression, moreover they found a positive correlation between H19 LncRNA expression level and HER2 / neu indicating that H19 is a potential regulator of proliferation in the HER2/ neu enriched subtype.

This study showed that plasma H19 had much higher sensitivity (83.3%) and specificity (86.1%) than CA15.3 in differentiating breast cancer cases from controls thus it can be used as potential diagnostic marker in breast cancer.

These results agreed with **Zhang et al (6)** who found H19 had higher specificity 86.7% than CA15.3 (60%).

The study **Zhong et al (19)** reported that the sensitivity of H19 was (87.0%) and the specificity was (70.6%) which were significantly higher than the sensitivity of CA15-3 and CEA (67.2% and 66.5% respectively) and specificity of them (58,3% and 52.7% respectively), they also calculated the combined diagnostic value of CA15.3 and CEA; sensitivity and specificity were (78.4% and 67.3% respectively). The results indicated that exosomal H19 was superior to the traditional markers in BC diagnosis. Furthermore, sensitivity and specificity for the combination of three markers was (89.2% and 76.3%respectively), thus implying exosomal H19 to be an appropriate diagnostic marker for BC.

In this study, ROC curve analysis was done for H19 and CA15.3 to discriminate between TNM (I/II) and (III/IV) stages of breast cancer patient revealing better performance of H19 as the AUC was (0.831), sensitivity 74.3% and specificity 82.8%, however the AUC of Ca15-3 was (0.7990), sensitivity was 71.4% and specificity was 75.9%.

Conclusion,

Plasma H19 expression levels were up regulated in patients with BC. Moreover, long non coding RNA H19 showed better performance than CA15-3 in diagnosis and prognosis of breast cancer differentiating early stages I/II from late stages III/IV. Thus it could be used as promising biomarker in diagnosis and prognosis of breast cancer.

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