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Long noncoding RNA H19 in breast cancer; impact of its gene expression and rs217727 single nucleotide polymorphism distribution

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Abstract

Breast cancer (BC), the most common women malignancy, comes second for cancer-related deaths. Roles of long non-coding RNA H19 (lncH19) re-expression and rs217727 SNP in BC is still obscure. We aimed at evaluating lncH19 expression**,** determining rs217727 distribution and assessing its impact on gene expression among BC females and its possible utilization as a marker for BC diagnosis or prognosis. This study performed on 200 females; 100 BC females, 50 benign breast lesion (BBL) females and 50 healthy controls. LncH19 polymorphism (rs217727) was genotyped with restriction fragment-length polymorphism. Relative quantitation of lncH19 expression done by real-time PCR. LncH19 rs217727 distribution showed significant increases in frequencies of homozygous TT variant genotype of co-dominant model, CT+TT dominant model and T allele in BC versus controls. There was significantly increased T allele frequency in BC versus BBL. LncH19 expression significantly increased in BC versus BBL and HC. LncH19 upregulated in BC with metastasis which suggests its prognostic value. ROC curve analysis showed the possibility of the lncH19 for being a BC diagnostic biomarker. The diagnostic performance for expression showed 85% sensitivity and 83% specificity. LncH19 rs217727 T allele carries excess BC risk. LncH19 expression might be a diagnostic and prognostic marker for BC.

Keywords: Breast cancer, lncRNA, lncH19, polymorphism, gene expression.

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Introduction

Breast cancer (BC) is the most commonly detected cancer in females. BC comes second among causes for cancer-related death in females [1], accounting for about 23% of all cancer deaths in them [2]. Being the most prevalent among Egyptian women [3], Egypt is the second leading country in terms of absolute burden with a mortality-to-incidence ratio accounting for 40% according to GLOBOCAN 2018 [4].

BC development is a multifactorial and multi-step process. Gene variation and irregularly expressed oncogenes and anti-oncogenes have been involved in tumor commencement and progression. Genetic contribution also occurs via BRCA1/2, HER2, epidermal growth factor receptor (EGFR), c-Myc and Ras gene family. Other risk factors do occur including longevity, medical history of family members and factors related to reproduction like early menarche, delayed menopause, advanced maternal age at first pregnancy and decreased parity. Both endogenous and exogenous estrogens are related to BC risk. Modern lifestyles like extravagant alcohol consumption and immoderate fatty diet augments the BC risk [5].

The reconnaissance of human genome has displayed that just 2% of the whole human genes code for protein while the other 98% are non-coding. Based on length, non-coding RNAs has many classes like long non-coding RNA (lncRNA), small nuclear RNA, small nucleolar RNA, micro RNA (miRNA), piwi interacting RNA, and small interfering RNA. Non-coding RNAs have noteworthy diverse biological roles [6]. The lncRNAs, composed of more than 200 bp in length, have important roles in many pathophysiological processes occurring in cancer development as cellular proliferation, differentiation, metastasis, angiogenesis, and drug resistance [7]. LncRNA display tissue and stage specific gene expression, showing high sensitivity and specificity, especially in hormone sensitive cancers [8]. LncRNAs are distinguished by being stable (~3.5 hours) in biological samples like blood, on the contrary to mRNAs that are unstable (< 2 hours) in such samples

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[9]. A condition that help detect lncRNAs using liquid samples by polymerase chain reaction (PCR) that take about 2 hours to have results in contrast to immune histochemistry (IHC), that takes nearly two days or more [10].

Long non-coding RNA H19 (lncH19) was the headmost detected. Its locus is 11p15.5. It has 5 exons and 4 small introns [11]. The lncH19 is an oncofetal imprinted gene expressed exclusively from the maternal allele and is one of the highest expressed genes during embryogenesis [7, 12]. It is normally down-regulated after birth [13]. Its abnormal expression can cause numerous human illnesses [14], especially cancers [15]. Gene polymorphisms of lncH19, including the functional rs217727 single nucleotide polymorphism (SNP) positioned to exon 5 of lncH19 gene with C to T, may set lncH19 expression and function and are linked to various diseases [16]. The biological effect and the possible molecular behaviour of lncH19 in BC are still obscure [17].

Early detection of BC allow the commencement of early treatment, so that advanced stages are averted and the incidence of complications could be decreased especially the serious ones as distant metastasis. This could decrease the mortality and improve the survival of patients and the quality of life for them [18].

Better prognosis of BC is yet connected to detection at early stages, emphasizing on improved screening strategies that respect time factor [19].

The current study aimed to evaluate the level of lncH19 expression, determine the distribution of rs217727 SNP and assess the impact of this polymorphism on the gene expression among all the study groups.

2. Materials and methods:

2.1. Materials:

This study (case-control) was executed on two hundred Egyptian women; including fifty apparent healthy controls (HC), fifty women suffering benign breast lesion (BBL) and hundred women with BC. The sample size was calculated using G*power software version 3.1.9.2 based on a previous study by Mohammadi et al. [20] who reported a large effect size of lncH19 gene expression between the studied groups. The sample size needed to detect a large effect size (d = 0.8) is 45 per group. The sample size was increased to 50 per group to compensate for possible laboratory failures. Alpha and power were adjusted at 0.05 and 0.95, respectively.

All participants were unrelated Egyptian residents in Qalyubia governorate. The BBL and BC cases were selected from General Surgery Department & Oncology Unit of General Medicine Department, Benha University Hospitals. Entire history taking, complete clinical examination and radiological assessment (breast US and mammography) were applied to all participants. Diagnosis of BBL and BC cases was confirmed by histopathology. Clinico-pathological characteristics of cases was obtained from medical reports. Pathological data of BC cases, including estrogen and progesterone receptors (ER & PR), and human epidermal growth factor receptor-2 (HER-2) was obtained via IHC reports. HC have no breast lesions either clinically & radiologically and were randomly selected from attendants of Benha University Hospital for routine health checkups. The molecular analysis of lncH19 gene expression and polymorphism for all participants was performed in the Molecular Biology Unit, Benha Faculty of Medicine.

Written informed consents were gained former to involvement in the study either from subjects included themselves or their next of kin. An assent was gained from Ethical Committee of Benha Faculty of Medicine study no. MSc.15.1.2019.

2.2. Methods:

2.2.1. Specimen collection:

About two ml of venous blood / subject were acquired on ethylene diamine tetra-acetic acid (EDTA), divided in two Eppendorf tubes (one for genotyping and the other for expression). EDTA-blood samples were kept at -80^{o}C till being extracted.

2.2.2. Genotyping of lncH19 SNP (rs217727):

It was detected by restriction fragment length polymorphism (RFLP) as follow:

1- **DNA extraction:** Genomic DNA was extracted using 100 µl EDTA-blood by Quick-gDNA Miniprep kit (Zymo research, USA) in line with steps settled by the manufacturer. The optical density (OD) of the DNA extract at wavelengths 260 and 280 nm was assessed by Nanodrop Spectrophotometer 2000 (Thermo-Fisher Scientific, Wilmington, USA). Pure DNA extract appeared to have an OD (260/280) from 1.7 to 2.0 [21].

2- **Genomic DNA amplification**: PCR was implemented in a 25 µl reaction by primers for lncH19 rs217727 [16]. The sequences of rs217727 primers are listed in table 1. Amplification was executed in Veriti™ Thermal Cycler (Applied Biosystems). PCR mix comprised 12.5 µl Easy taq PCR SuperMix (Transgen biotech, China) 1 µl FP, 1 µl RP, 5 µl DNA template and completed up to 25 µl by water free of nucleases. The thermal cycling program contained initial denaturation (5 min at 95°C), 35

cycles (denaturation; 30 sec at 95°C, annealing; 30 sec at 60°C, and extension; 1 min at 72°C) and then final extension (5 min at 72°C). Gel electrophoresis for amplified yield was done on 3% agarose gel containing ethidium bromide stain (0.3 ug.ml^{-1}) to detect the molecular weight of bands. For each sample, the amplified yield (10 µl) and 100 base pair ladder (5 µl) were mixed well and run on the gel. The resulting bands (prior digestion) were visualized through UV trans-illumination (254 nm) and photographed by a digital camera (8 mega pixel). Photos were analyzed through computer software (Alpha InoTech Gel Documentation System). Prior-digestion bands were seen at 247 bp. Figure 1-A

3- **Digestion by RsrII restriction enzyme (RE):** The RE was obtained from New England Biolabs (England). A fifty µl reaction was conducted / a sample. The digestion mixture contained PCR products (10 µl), 1µl RsrII RE (1 unit), 5µl 10X buffer and 34 µl water free of nucleases. Mixtures were incubated an hour at 37°C followed by twenty minutes' inactivation at 65°C. Gel electrophoresis for digested DNA fragments was applied with the same conditions mentioned above for the pre-digestion bands. For the after-digestion bands; the uncut T allele appeared as a single 247 bp fragment, while the C allele (cut) appeared as two bands (221 bp & 26 bp). The smaller one (26 bp) did not appear on the gel (small sized). Figure 1-B

2.2.3. Measurement of lncH19 gene expression: it was performed as follow:

1- **Total RNA Extraction:** Extraction from a 100 µl EDTA-blood was executed by means of Total RNA Purification Kit (Jena Bioscience, Germany) in line with steps settled by the manufacturer. Nucleic acid quantification was detected though Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Refined RNA extracts was defined as a 260/280 OD ratio ranges from of 1.9 to 2.3 [21].

2- **Reverse transcription (RT):** cDNA was executed in a Veriti™ Thermal Cycler (Applied Biosystems), by Maxime RT PreMix (random primer) Kit (Intron Biotechnology, Korea). To an RT tube provided; 5 µl RNA template and 15 µl water (free of nucleases) were appended. Then tubes incubated an hour at 42ºC followed by 10 minutes of RTase inactivation at 85ºC.

3- **Relative quantitation (RQ) of lncH19 expression:** It was implemented by Hera Sybr Green qPCR kit (Willowfort, UK). Singleplex reactions were done. The sequences of primers used for expression of the lncH19 gene [22] and the human U6 housekeeping gene [23] are listed in table 1. To avoid bias in RQ, the use of a suitable housekeeping gene (human U6) for data normalization is essential. Being highly conserved and due to its lower M value, human U6 is commonly used for normalization of different types of mRNA [24] including lncRNA [25]. Each reaction comprised 10µl Hera Sybr master mix (2X), 1µl FP, 1 µl RP, 4 µl cDNA and up to 20 µl by water free of nucleases. Amplification took place in Stepone Real-Time Cycler (Applied Biosystem, Singapore). At first, holding stage (95ºC / 10 min) was executed then cycling was applied (40 cycles; denaturation for 14 sec at 95ºC, annealing / extension for 1 min at 58ºC). Melting curve ensured specificity.

4- **Data analysis of lncH19 expression:** In means of stepone software (v2.2.2.), lncH19 expression in HC was defined as 1. RQ of target gene expression was normalized to that of human U6. ΔCT was set by subtracting threshold cycle (CT) of U6 from CT of lncH19. ΔΔCT was set by sub-tracting ΔCT of HC (calibrator group) from ΔCT of test group (BBL or BC). Gene expression fold changes were calculated through $2^{-\Delta\Delta CT}$ equation [26].

Table 1: The sequences of primers used for polymorphism and gene expression in this study

Primers
IncH19 polymorphism
rs217727
FP: **5'**-ACTCACGAATCGGCTCTGGAAGGTG-**3'**
RP: **5'**-ATGTGGTGGCTGGTGGTCAACGGT-**3'**
IncH19 gene expression
Human lncH19
FP: **5'**-ATCGGTGCCTCAGCGTTCGG-**3'**
RP: **5'**-CTGTCCTCGCCGTCACACCG-**3'**
Human U6
FP: **5'**-GTGCTCGCTTCGGCAGCA-**3'**
RP: **5'**-CAAAATATGGAACGCTTC-**3'**

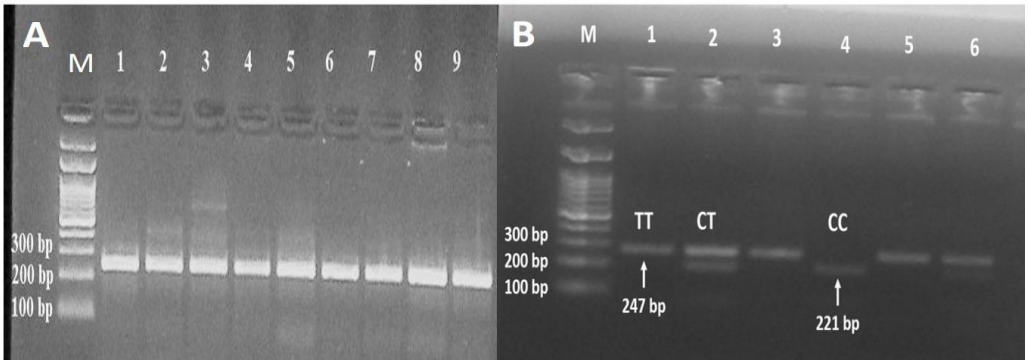

Figure 1: Photos for gel electrophoresis of lncH19 rs217727. A: Bands before digestion, B: Bands after digestion; RFLP by RsII RE [(TT → 247 bp), (CC → 221 bp & 26 bp), (CT → 247, 221 & 26 bp)]; the smallest band (26 bp) did not appear on the gel.

2.3. Statistical analysis:

Statistical analysis was implemented via Microsoft Office Excel (2016), Statistics Package for Social Sciences (IBM SPSS statistics version 20) and medical calculation (MEDCALC) package. Qualitative data were shown as number (n.) and percentage (%) and was tested by Chi-square test (X^2) or Fisher's exact test (FET) as convenient. Quantitative data was shown as mean ± standard deviation (SD) if normally distributed or median and interquartile range (IQR) if abnormally distributed. Student "t" test and analysis of variance (ANOVA) test were applied to compare the means of normally distributed quantitative variables; "t" for 2 groups and ANOVA for more than 2 groups. Mann Whitney U (MWU) test was utilized for abnormally distributed quantitative variables between two groups. $P < 0.05$ was significant. Odd ratio (OR) and confidence interval (CI) were calculated for SNP distribution. Receiver operator characteristic (ROC) curve was plotted for gene expression. For gene polymorphism, all groups of study were consistent with Hardy-Weinberg equilibrium (HWE) [X^2= 0.009, p=0.92 for HC, X^2= 0.003, p= 0.96 for BBL and X^2= 0.17, p=0.68 for BC].

3. Results

The homozygous TT genotype appeared as a single band (247 bp) on the electrophoresis gel. The homozygous CC genotype gave two bands (221 bp & 26 bp). The heterozygous CT genotype gave three bands (247 bp, 221 bp & 26 bp). The 26 bp band didn't appear on the gel due to its small molecular weight (Figure 1).

The current study showed non-significant differences in demographic characteristics of studied groups (Table 2).

The overall genotype and allele frequencies for lncH19 rs217727 polymorphism in BC, BBL and controls were shown in table 3. There were significant increases in the frequencies of the homozygous TT variant genotype of the co-dominant model (p = 0.02, OR = 4.29, 95% CI= 1.14-16.08), the CT+TT dominant model (p = 0.03, OR= 2.17, 95% CI = 1.09-4.32) and the T allele frequency (p = 0.01, OR = 2.00, 95% CI = 1.17-3.41) in BC group versus controls. Moreover, there was significant increased T allele frequency in BC compared to BBL (p = 0.04, OR = 1.71, 95% CI = 1.02-2.88) (Table 3).

The clinico-pathological criteria of BC regarding staging, grading, LN status, hormonal status & distant metastasis were shown in Figure 2-A. Gene expression levels of lncH19 showed considerable excess in BC versus BBL and HC (p value < 0.001 for both) (Figure 2-B). Within HC, the lncH19 expression didn't show any difference between the three genotypes (p = 0.72). Within the BBL group, there was non-significant increase in both CT and TT genotypes (p = 0.15). However, the BC showed significantly increased lncH19 expression in the CT and TT genotypes versus the CC genotype (p < 0.001 for both) (Figure 2-C). On stratifying BC cases by the clinico-pathological criteria (tumor grading, staging, hormonal status and the occurrence of distant metastasis, non-significant differences (p > 0.05) in lncH19 gene expression occurred apart from the significant level (p = 0.043) in BC with distant metastasis (Figure 2-D), a condition which suggests its use as a prognostic factor for BC. The ROC curve analysis showed the possibility of lncH19 for being a diagnostic biomarker of BC (Figure 2-E)

There were no significant differences regarding the demographic and clinical characteristics within BC when stratified by rs217727 genotypes (Table 4).

The diagnostic performance of lncH19 gene expression as a diagnostic biomarker for BC showed 85% sensitivity, 83% specificity with area under the curve (AUC) of 0.920 – 0.977 for a cutoff level off ≥ 1.35 fold increase (Table 5).

Table 2: Demographic characteristics of studied groups

Variable		HC (n. = 50)	BBL (n. = 50)	BC (n. = 100)	Test	P
		Mean ± SD or n. (%)				
Age (Years)		48.32 ± 12.02	45.5 ± 12.28	49.45 ± 10.71	F= 1.99	0.14
Age at menarche (Years)		13.02 ± 1.66	12.92 ± 1.35	12.95 ± 1.33	F= 0.07	0.94
Age at menopause (Years) (postmenopausal)		48.45 ± 4.69	46.57 ± 10.42	49.86 ± 4.62	F= 2.75	0.07
Marital status Single / married		10 (20) / 40 (80)	10 (20) / 40 (80)	15 (15) / 85 (85)	X^2= 0.87	0.65
Number of pregnancies Nulligravida Primigravida Multigravida		10 (20) 3 (6) 37 (74)	12 (24) 5 (10) 33 (66)	19 (19) 8 (8) 73 (73)	FET= 1.3	0.88
Number of abortions (One / two)		3 (6) / 5 (10)	3 (6) / 4 (8)	7 (7) / 11 (11)	FET= 0.46	0.99
Menopausal status (Pre / Post)		24 (48) /26 (52)	27 (54) / 23 (46)	47 (47) / 53 (53)	X^2= 0.65	0.42
Breast feeding		30 (60)	30 (60)	66 (66)	X^2= 0.77	0.68
Family history		12 (24)	14 (28)	26 (26)	X^2= 0.21	0.90
Systemic diseases	**DM HTN DM & HTN**	10 (20) 14 (28) 8 (16)	13 (26) 11 (22) 7 (14)	27 (27) 36 (36) 16 (16)	X^2= 7.5	0.28
Smoking		3 (6)	3 (6)	7 (7)	FET= 0.12	1.0
Contraception	**Pills / IUD**	13 (26) / 21 (42)	11 (22) / 22 (44)	23 (23) / 54 (54)	X^2= 3.28	0.51

HC: healthy controls, BBL: benign breast lesion, BC: breast cancer, DM: diabetes mellitus, HTN: hypertension, IUD: intra-uterine device, F: ANOVA (analysis of variance), X^2: Chi-square; FET: Fisher's exact test

Table 3: Distribution of genotypic & allelic frequencies of lncH19 rs217727 in the studied groups

Variable	HC (n.=50)	BBL (n.=50)	P	OR (95%CI)	HC (n.=50)	BC (n.=100)	P	OR (95%CI)	BBL (n.=50)	BC (n.=100)	p	OR (95%CI)
	n. (%)				n. (%)				n. (%)			
Co-dominant model												
CC	28 (56)	26 (52)	Reference		28 (56)	37 (37)	Reference		26 (52)	37 (37)	Reference	
CT	19 (38)	20 (40)	0.77	1.13 (0.50-2.59)	19 (38)	46 (46)	0.10	1.83 (0.89-3.79)	20 (40)	46 (46)	0.19	1.62 (0.78-3.34)
TT	3 (6)	4 (8)	0.65	1.44 (0.29-7.04)	3 (6)	17 (17)	**0.02***	**4.29 (1.14-16.08)**	4 (8)	17 (17)	0.07	2.99 (0.90-9.91)
Dominant model												
CC	28 (56)	26 (52)	Reference		28 (56)	37 (37)	Reference		26 (52)	37 (37)	Reference	
CT+TT	22 (44)	24 (48)	0.69	1.18 (0.54-2.58)	22 (44)	63 (63)	**0.03***	**2.17 (1.09-4.32)**	24 (48)	63 (63)	0.08	1.85 (0.93-3.67)
Recessive model												
CC+CT	47 (94)	46 (92)	Reference		47 (94)	83 (83)	Reference		46 (92)	83 (83)	Reference	
TT	3 (6)	4 (8)	0.70	1.36 (0.29-6.43)	3 (6)	17 (17)	0.06	3.21 (0.89-11.52)	4 (8)	17 (17)	0.13	2.36 (0.75-7.42)
Over-dominant model												
CC+TT	31 (62)	30 (60)	Reference		31 (62)	54 (54)	Reference		30 (60)	54 (54)	Reference	
CT	19 (38)	20 (40)	0.84	1.09 (0.49-2.43)	19 (38)	46 (46)	0.35	1.39 (0.69-2.78)	20 (40)	46 (46)	0.49	1.28 (0.64-2.55)
Allele frequencies												
C	75 (75)	72 (72)	Reference		75 (75)	120 (60)	Reference		72 (72)	120 (60)	Reference	
T	25 (25)	28 (28)	0.63	1.17 (0.62-2.19)	25 (25)	80 (40)	**0.01***	**2.00 (1.17-3.41)**	28 (28)	80 (40)	**0.04***	**1.71 (1.02-2.88)**

HC: healthy controls, BBL: benign breast lesion, BC: breast cancer
X^2: Chi-square was used; OR: odd ratio, CI: confidence interval, p > 0.05: non-significant difference, *: significant

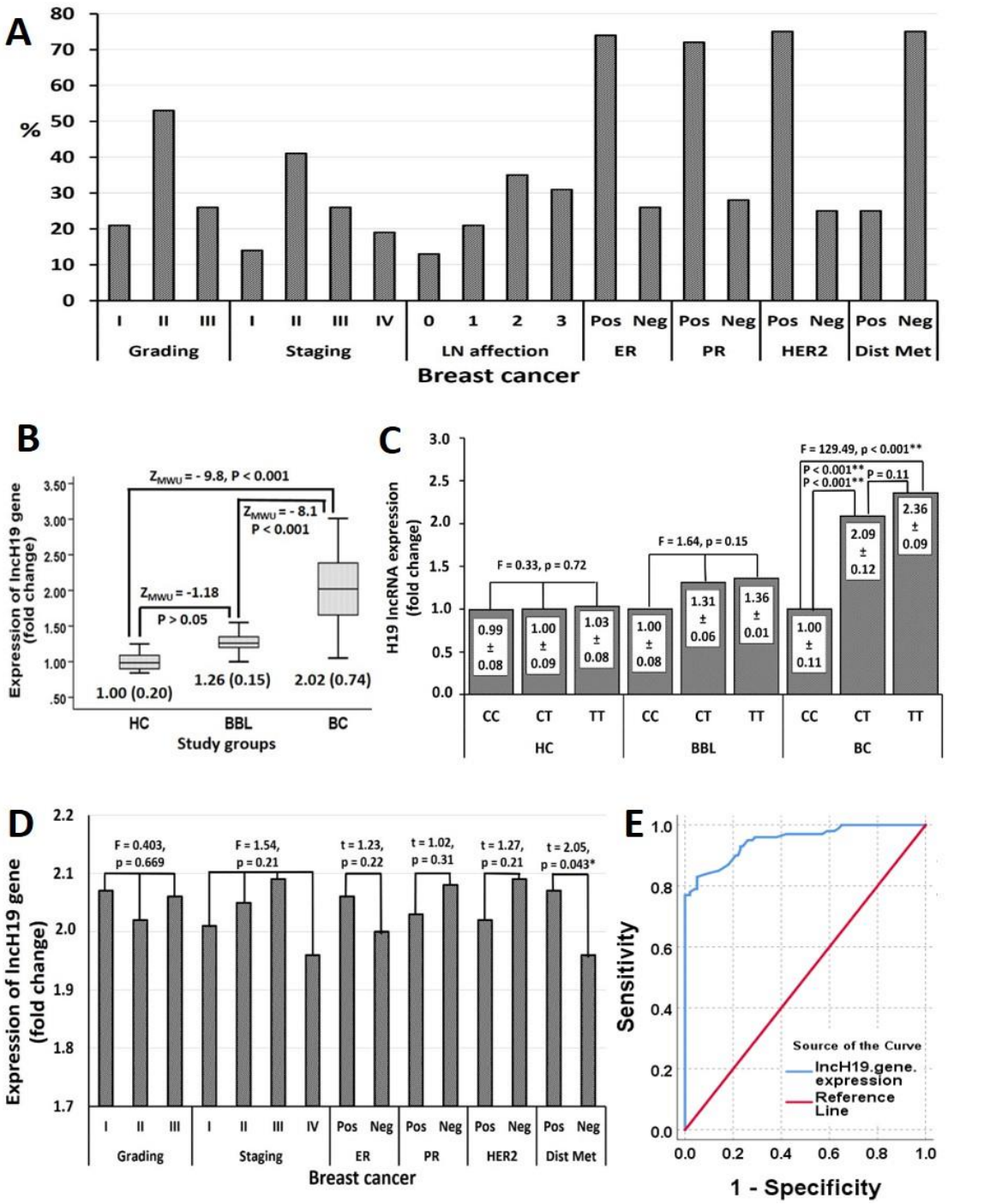

Figure 2: A: Clinico-pathological parameters in the BC group, B: lncH19 gene expression among the studied groups expressed as median and IQR, C: lncH19 gene expression in all groups stratified by rs217727 genotypes expressed as mean ± SD, D: lncH19 gene expression in BC according to clinico-pathological parameters, E: ROC curve analysis for diagnosis of breast cancer.

LN: lymph nodes, ER: estrogen receptors, PR: progesterone receptors, Her2: human epidermal growth factor receptor 2, Pos: positive, Neg: negative, Dis Met: distant metastasis, HC: healthy controls, BBL: benign breast lesion, BC: breast cancer, ZMWU: z value of Mnn Whitney test, F: ANOVA (analysis of variance), t: student "t" test, *: significant, **: high significant

Table 4: Demographic and clinical characteristics of breast cancer by lncH19 rs217727 genotypes

Variables		CC (n. = 37)	CT (n. = 46)	TT (n. = 17)	Test	p
Genotype		**Mean ± SD or n. (%)**				
Age (Years)		50.32 ± 11.42	49.3 ± 11.15	47.94 ± 7.9	F= 0.29	0.75
Age at menarche (Years)		13.11 ± 1.35	12.96 ± 1.30	12.59 ± 1.37	F= 0.89	0.41
Age at menopause (Years) (postmenopausal)		50.04 ± 5.05	50.13 ± 4.19	48.75 ± 5.01	F= 0.41	0.67
Marital status Single / married		6 (16.2) / 31 (83.8)	8 (17.4) / 38 (82.6)	1 (5.9) / 16 (94.1)	X^2= 1.36	0.51
Number of pregnancies						
Nulligravida		8 (21.6)	10 (21.7)	1 (5.9)	FET= 2.59	0.67
Primigravida		3 (8.1)	4 (8.7)	1 (5.9)		
Multigravida		26 (70.3)	32 (69.6)	15 (88.2)		
Number of abortions (One / two)		3 (8.1) / 3 (8.1)	2 (4.3) / 5 (10.9)	2 (11.8) / 3 (17.6)	FET= 2.86	0.58
Menopausal status (Pre / Post)		17 (45.9) / 20 (54.1)	23 (50) / 23 (50)	7 (41.2) / 10 (58.8)	X^2= 0.41	0.81
Breast feeding		27 (73)	28 (60.9)	11 (64.7)	X^2= 1.35	0.51
Family history		10 (27)	12 (26.1)	4 (23.5)	X^2= 0.07	0.96
Systemic diseases	**DM**	11 (29.7)	8 (17.4)	8 (47.1)	FET= 7.27	0.29
	HTN	15 (40.5)	17 (37)	4 (23.5)		
	DM & HTN	5 (13.5)	8 (17.4)	3 (17.6)		
Smoking		3 (8.1)	2 (4.3)	2 (11.8)	FET= 1.49	0.52
Contraception Pills / Intra-uterine device		7 (18.9) / 21 (56.8)	12 (26.1) / 22 (47.8)	4 (23.5) / 11 (64.7)	FET= 2.31	0.69
ER status (Pos/Neg)		26 (70.3) / 11 (29.7)	33 (71.7) / 13 (28.3)	15 (88.2) / 2 (11.8)	X^2= 2.18	0.34
PR status (Pos/Neg)		27 (73) / 10 (27)	35 (76.1) / 11 (23.9)	10 (58.8) / 7 (41.2)	X^2= 1.86	0.39
HER2 status (Pos/Neg)		28 (75.7) / 9 (24.3)	36 (78.3) / 10 (21.7)	11 (64.7) / 6 (35.3)	X^2= 1.23	0.54
Distant metastasis (Pos/Neg)		11 (29.7) / 26 (70.3)	12 (26.1) / 34 (73.9)	2 (11.8) / 15 (88.2)	X^2= 2.06	0.36

DM: diabetes mellitus, HTN: hypertension, ER: estrogen receptors, PR: progesterone receptors, Her2/neo: human epidermal growth factor receptor 2, Pos: positive, Neg: negative, F: ANOVA (analysis of variance), X^2: Chi-square; FET: Fisher's exact test

Table 5: Performance of lncH19 gene expression for breast cancer diagnosis

lncH19 gene expression level (fold change)	Sensitivity	Specificity	PPV	NPV	AUC	95% CI	p
Cut-off ≥ 1.35	85%	83%	83.3	84.7	0.949	0.920 – 0.977	< 0.001**

PPV: positive predictive value, NPV: negative predictive value, AUC: area under curve, CI: confidence interval

4. Discussion

LncH19 has key functions in many physiological and pathological processes. During embryonic development, it acts essentially in angiogenesis at the maternal-fetal interface, considerably affecting the exchange of nutrients and oxygen between the mother and foetus [27]. Pathologically, various studies have shown its role in many illnesses, especially cancer [28]. Being seriously implicated in cell cycle coordination, lncRNAs are substantial governors of tissue pathology and illness. Gene polymorphism of which may alter the structure, steadiness, and expression level of lncRNA [29]. The lncRNAs are precursors of microRNAs (miRNAs) or work as competing endogenous RNAs (ceRNAs) that react with miRNAs [30].

The present study explored the efficacy of lncH19 as likely molecular noninvasive tumor markers for diagnosis of BC among Egyptian females, evaluated the interactions between lncH19 expression and rs217727 distribution and the risk of BC in Egyptian females and also studied the impact of this SNP distribution on the expression of lncH19 in BC.

Our results concerning the demographic data between the studied groups were supported by Xia et al. [31] who found insignificant variations between their studied BC women and control subjects.

In consistence with our results, T allele carriers have a significantly promoted risk for BC development [17, 32]. In addition, Wang et al. and Li et al. [33, 34] found considerable enhanced relevance of rs217727 to BC susceptibility in allele model and different genotype models. The rs217727 was also connected to oral squamous cell carcinoma (OSCC) risk in Iranian population, where the T allele frequency yielded a significant increase in OSCC in the co-dominant, dominant, and recessive models [35]. Moreover, rs217727 was linked to the risk of ER-positivity of BC [36].

In contrast to our results, Mathias et al., [37] negated the presence of an association between the rs217727 and BC. Insignificant contributions of rs217727 SNP to the susceptibility of BC in Chinese [31] and Iranian women [16] were reported. Lv et al. [38] stated that lncH19 SNPs other than rs217727 were linked to the overall cancer risk. A recent meta-analysis manifested that 5 SNPs on H19 gene including rs217727 had no influence on the overall cancer susceptibility, they did not promote lncH19 as a perfect marker for cancer diagnosis and treatment despite being believed to be a possible new remediation target for mending of tumor resistance to therapy [11]. The rs217727 T allele was joined to a considerable lowered hepatoblastoma risk as well [39].

Our results yielded non-significant differences of the demographic data on stratifying BC cases by genotypes. These findings were corresponding to Xia et al. [31] and Hassanzarei et al. [32]. Despite, Lin et al. [17] reported that the T allele carrier state in BC was significantly associated with younger age, and overdue menarche, overdue menopause, first live birth at early age, and fewer pregnancies.

Our results concerning the excess expression of lncH19 in BC compared to benign and control groups was in agreement with Zhong et al. [40] and Vennin et al. [41], who identified that lncH19 increases cell tumorigenic capacities in vitro and in vivo and acts as an oncogene by masking methylation site and H19 promoter regulates expression of the H19/IGF2 imprinted locus.

Expression of lncH19 gene has been tested as a particular biomarker for cancer detection and a fantastic remedy target as well [7, 42, 43]. LncH19 expression has been evidenced to be deregulated in human cancers, a condition that is reflected on cancer biology by different mechanisms like excess miRNA expression as miR-675, miRNA sponging and epigenetic modifications [44].

The miR-675 is derived from lncH19 giving rise to miR-675-5p and miR-675-3p. In human BC, c-Cbl and Cbl-b are 2 members of ubiquitin ligase E3 family. Overexpression of miR-675-5p represses c-Cbl and Cbl-b and therefore enhanced the constancy and action of EGFR and c-Met in BC cell line. In addition, miR-675 shared in repression of E-cadherin via up-regulation of transcription factor Slug and so raised invasion and metastasis in BC [12]. Mature miRNA aggregate with RNA-induced silencing complex (RISC). LncH19 expression may increase or decrease in cancers [45]. The tumor suppressor RB is a miR-675 target. In human BC cells, lncH19 sponges miR-152 prohibiting its tumor restraining action, so proliferation and migration of cancer do occur [12].

LncH19 reinforces cell proliferation via diverse mechanisms. LncH19 promoter is stimulated by E2F1 affecting the G1-S transition in BC cells [46]. As an estrogen-inducible gene; H19 has an essential role in surviving and estrogen-induced propagation of MCF-7 cells [47]. Si et al. [48] found that lncH19 weakened the cell apoptosis through the repression of pro-apoptotic transcription of genes. The aggressive phenotype of BC cells manifested by the boosted tumor growth and metastasis in vivo might be due to the excess expression of H19/miR-675 [41] and the suggested crosstalk between this lncRNA and miRNAs that probably influence BC initiation and progression. Moreover, such interaction tangles the way of post-transcription regulation of gene expression [30]. LncH19 was up-

regulated in plasma of BC [49], especially the HER2-positive [30] and the triple negative BC [50]. LncH19 can sponge many tumor suppressor miRNAs as miR-152 affecting the miR-152/DNA methyltransferase 1 (DNMT1) axis, let-7a which functions via connecting the 3′UTR of CC chemokine receptor 7 (CCR7) mRNA and miR-200b which acts by diverse pathways as inhibition of Rac1, restraint of angiogenesis, and suppression of epithelial to mesenchymal transition (EMT). LncH19 knockdown increased the DNMT3B-interposed methylation of Nctc1, a gene that encodes lncRNAs, within Igf2-H19-Nctc1 locus. So, lncH19 modified the DNA methylation and yielded breast tumorigenesis. Depressed lncH19 expression also represses cell proliferation, invasion and migration, thus enhancing cell cycle arrest and apoptosis via regulating miR-138 and SOX4. LncH19 is increased in BC tissues and inversely proportionate to miR-138 expression [12, 51, 52].

LncH19 can act on chromatin assembly by means of recruiting chromatin modulating complex PRC2 and after-transcription control as a miRNA decoys segregating miR-106a and miR-let7 or as a progenitor for miR-675-5p and miR-675-3p. LncH19 react with p53 and prohibits the tumor inhibiting protein function as well. In addition, potential base pairing between lncH19 and the antisense transcripts 91H and HOTS may be of biologic consequences [29, 53].

LncRNAs modify the expression of many tumor inhibiting genes and oncogenes. Retinoblastoma (Rb) gene, tumor-inhibiting, is a negative regulator of lncH19 gene [54], however, c-Myc oncogene enhanced lncH19 expression by recruitment of histone acetyltransferase onto lncH19 promoter increasing the proliferation of BC cells [55]. LncRNAs also modify the effectiveness of cancer-associated signaling axes as lncH19/miR-15b/CDC42 and lncH19/miR-326/TWIST1 axes. Silenced lncH19 suppresses proliferation, peregrination and invasiveness of malignant cells, while it encourages apoptosis by means of miR-15b/CDC42/PAK1 pathway [56].

H19 can also set autophagy and ferroptosis, through the regulation of lipid reactive oxygen species so that lncH19 is being referred to as a possible therapeutic target for BC [57].

Many researchers have recognized deregulated expression of lncH19 in multiple human malignancies, like leukemia, lymphoma, colorectal, gastric, pancreatic, glioma and ovarian cancers [58]. Moreover, rs217727 is tightly associated with cancer susceptibility [50]. LncH19 expression was enhanced in hepatocellular carcinoma (HCC) and was inhibited following partial or entire response to therapy. LncH19 could be an emerging biomarker of HCC [44, 59]. LncH19 expression was positively associated with infiltration level of many immune cells encompassing T-helper cells (CD4+ and CD8+), B cells, dendritic cells, neutrophils and macrophages in thyroid cancer [60]. LncH19 was overexpressed in fecal colonocytes of colorectal carcinoma versus controls, a finding that supported the use of lncH19 for early detection [61] and it was also linked to the advanced stage of cancer colon and was a significant risk factor for decreased recurrence-free survival. LncH19 expression may be potentially used for forthcoming prognostic and therapeutic purposes as well [62, 63].

The present work disclosed that lncH19 expression presented significant rise in TT & CT genotypes in BC versus the other 2 groups. This finding agreed with Lin et al. [17] who manifested that lncH19 expression was enhanced in BC tissue with more significant increment in CT or TT genotypes of rs217727 versus CC genotype within the BC group. These results may be explained as rs217727 SNP of lncH19 may affect the promoter activity (transcription regulation), mRNA conformation (stability), and translational efficiency. The effect on the translational efficiency may alter the lncH19 structure, and therefore the function of lncH19 [17]. It may also influence the function of lncRNA via lncRNA/miRNA interaction [35] altering miRNA target loci and change of the RNA secondary structure [45].

Our results elucidated that expression & polymorphism of lncH19 were insignificantly linked to staging & grading of BC, but significantly associated with metastasis pointing out that it may be a prospective biomarker for prognosis. The overexpression of lncH19 in metastatic versus the non-metastatic BC went coinciding with Zhong et al. [40] and Sun et al. [47]. LncRNAs boost sending secondary tumors by working in various pro-metastatic junctures as the E-M transition, invasiveness, and peregrination and by affecting the metastatic cancer microenvironment through reacting with ribo-nucleoprotein complexes or straightway with different nucleic acids. LncRNA assemble with TGFβ-ZEB1/ZEB2 or nuclear factor-κB routes. Moreover, several lncRNAs may assist in differentiation of cancers with excess metastatic jeopardy and promote up-to-date remedy concepts. Several lncRNAs were shown to be possible incoming targets for antisense oligonucleotide therapy in animal models [64]. Metastasis promotion was advocated in breast tumor mouse models where lncH19 was particularly overexpressed in clones that were able seed metastases [64].

Loss of imprinting results in lncH19 re-expression that is linked to various phases of tumorigenesis proved by studies either on mice BC models or human cell lines. The various consequences

attributed to whether lncH19 can behave as a tumor promoting or inhibiting gene could be clarified through diversity in genetic dependencies of cancer and utilizing various models, (i.e. transgenic animals versus cell lines) [45]. LncH19 was assessed as an oncogene in choriocarcinoma [65] and as a tumor suppressor gene in osteosarcomagenesis [66].

Detection of cancer-related lncRNAs in body fluids is the incoming strategy not only for cancer diagnosis but also for earlier detection and for being therapeutic targets [65]. LncH19 in particular is a possible medication target for amendment of malignancy remediation reluctance in the future [67]**.**

5. Conclusion

Significant increases in the frequencies of TT genotype of the co-dominant model, the CT+TT dominant model and the T allele frequency in BC versus controls were observed. There is also significant increased T allele frequency in BC compared to BBL. Gene expression levels of lncH19 showed considerable excess in BC versus BBL and HC. Within groups, the lncH19 expression significantly increased in CT and TT genotypes versus the CC genotype only in BC but insignificantly increased in both CT and TT genotypes in BBL. Excess lncH19 expression occurred in BC with distant metastasis. ROC curve analysis and diagnostic performance of lncH19 gene expression showed 85% sensitivity and 83% specificity in BC. No significant differences were found regarding the demographic and clinical characteristics within BC when stratified by rs217727 genotypes.

LncH19 rs217727 T allele provides an up-growing hazard to BC. LncH19 expression provides a prospective diagnostic & prognostic biomarker for BC. We recommend more wide-based researches to emphasize the current findings and ensure possibility of being a future target for therapy and mending of treatment resistance.

6. References

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