

Assessment of Non Coding RNA Expression as a Potential Stem Cell Biomarker in Breast Cancer

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ABSTRACT

Background: worldwide, breast cancer is the most common malignancy among women and there is a deep need for precise novel methodologies for breast cancer (BC) diagnosis. Major advances in cancer control will be successfully achieved with early cancer detection. So, recent trends are going toward using circulating non-coding RNA as diagnostic tool for their critical role in cancer detection.

Aim: retrieve non coding RNA that is mechanistically linked to breast cancer stem cell with validation of the results in a group of breast cancer patients versus control groups to evaluate their usefulness as a potential biomarker in breast cancer diagnosis.

Patients and Methods: we retrieved LncRNA that is linked to stem cell differentiation and specific to BC utilizing bioinformatics tools. Then we validated this biomarker in serum of 30 patients with BC, 12 patients with benign breast lesion and 12 healthy volunteers using RT-qPCR. We evaluate the power of diagnosis of the serum profiling system using ROC curve analysis.

Results: hoxd antisense growth-associated long non coding RNA (HAGLR) had great sensitivity and specificity for differentiating BC from patients with benign breast lesion and also from healthy controls.

Conclusion: the chosen circulatory RNA based biomarker can be used as a potential diagnostic biomarker for BC. In addition it could be therapeutic target.

Keywords: breast cancer, long non coding RNA, bioinformatics, diagnosis.

INTRODUCTION

Breast cancer is the most frequent malignancy among women worldwide. It can be treated when diagnosed at its earliest stage. Every 3 minutes a female is diagnosed with BC globally, accounting to one million cases every year⁽¹⁾.

In Africa, there is a limited data about breast cancer incidence. The incidence rates for breast cancer between African countries vary considerably with the highest rates are in Gharbiah governorate (Egypt) [6078 cases reported in Egypt during the interval (1999-2007) and the lowest rates are in Ghana⁽²⁾.

When taking a clinical decision on BC treatment, Oncologists face an exceptionally hard task. Such hard task could be easier if there are strong diagnostic and prognostic factors, which guide the choices of treatment options⁽³⁾. Molecular technique, such non coding RNA (ncRNA) expression profile, has been used to improve BC diagnosis and to evaluate patient outcome and response to treatment⁽⁴⁾.

Recent evidence suggests that small populations of cancer stem cells can modify and influence neoplastic cells aggressiveness and behavior as well as response to therapy⁽⁵⁾. Many observations prove that BC ability to divide, progress and spread is based on this small subpopulation of cells with characteristics similar

to stem cells, known as breast cancer stem cells (BCSCs)⁽⁶⁾. Several markers have been reported for identification of BCSCs in many cancers, such as CD44, CD133, CD24, EpCAM, CD47 and ALDH1⁽⁷⁾.

Stem cells undergo two types of cell division (symmetric and asymmetric), producing similar stem cell colony or more differentiated cells. Moreover, stem cells remain in a state of quiescence in the tumor environment, facilitating the so-called kinetic resistance, whereby these quiescent cells are unaffected by agents that can cause DNA damaging or radiation in comparison with the more rapidly proliferating cells⁽⁸⁾. Also great drug resistance has been described for Cancer stem cells (CSCs) through different mechanisms such as drug effluxing⁽⁹⁾.

In recent years, lncRNA is emerging as an important player in the cancer paradigm. These non coding RNAs are often apparently des-regulated in many types of human cancers⁽¹⁰⁾. Accumulating evidences provide a mechanistic insight on how lncRNAs regulate important signaling pathways in cancer cells at the transcriptional, post-transcriptional and epigenetic levels⁽¹¹⁾. In addition to their effects on cancer cell growth, cell signaling and survival, lncRNA can modulate CSC behavior through the expression of pluripotency factors. The identification of lncRNAs that are linked to cancer stem cells differentiation and self-renewal provides new opportunities for cancer diagnosis and therapy⁽¹²⁾.

Among the oncogenic lncRNAs is *lincRNA-HOTAIR* (*HOX* antisense intergenic RNA), which is transcribed from the mammalian homeobox C (*HOXC*) gene locus on chromosome 12q13.13. *HOTAIR* is up-regulated in primary and metastatic breast cancer⁽¹³⁾.

Aim: the aim of the study is to retrieve non coding RNA that is mechanistically linked to breast cancer stem cell with validation of the results in a group of breast cancer patients versus control groups to evaluate their usefulness as a potential biomarker in breast cancer diagnosis.

PATIENTS AND METHODS

Patients and samples

Thirty BC patients participated in this study; diagnosis is done according to histopathological techniques. BC patients were classified according to clinical stages into; 12 (40%) stage I, 13(43.3%) stage II and 5 (16.7%) stage III carcinomas using the TNM classification *American Joint Committee on Cancer, 2016*⁽¹⁴⁾ and graded according to *American Cancer Society, 2017*⁽¹⁾. We collected venous blood samples from patients before any therapeutic interventions, including radical mastectomy or modified radical mastectomy, radiotherapy and chemotherapy. We analyzed the serum samples of 30 BC, 12 benign breast lesions and 12 healthy controls collected at general surgery department, Ain Shams University Hospital from June 2016 to November 2017.

Serum was obtained from each sample through centrifugation of venous blood samples collected from participants. All serum samples were kept at -80°C for further analysis.

Ethical and Approval Statements

The study was approved by the Ethics Board of Ain Shams University, Faculty of Medicine, Egypt and an informed written consent was obtained from each participant in the study.

Extraction of total RNA, including lncRNA

MiRNeasy® RNA isolation mini kit (Qiagen®, USA) was used to extract total RNA, including lncRNA from sera samples according to manufacturer's instructions. This is followed by reverse transcription of the extracted total RNA into cDNA with a miScriptRT II Kit (Qiagen®, USA) following the manufacturer protocol for sera/tissue samples) using Hybaid thermal cycler (Thermo Electron Waltham, MA).

Real time-PCR (qPCR) quantification of RNA based biomarker

LncRNA-HAGLR expression in participant's serum was assessed using RT² SYBR Green ROX qPCR Mastermix on Step One Plus™ System (Applied Biosystems Inc., Foster, CA).

GAPDH was used as an internal control. All the PCR primers were obtained from (Qiagen®, USA). Relative measurement of RNA based biomarker expression was figured using the $2^{-\Delta\Delta\text{Ct}}$ technique. For normalization of raw data we used GAPDH as a housekeeping gene as the invariant control for the samples and compared with a reference sample.

The PCR program for Syber green based QPCR was as follow: at first, initial activation step at 95°C for 10 min; after that 40 cycles of denaturation for 15 sec at 95°C ; then annealing for 30 sec at 55°C ; lastly, extension for 30 sec at 72°C .

The threshold cycle (Ct) value of each sample was calculated using StepOnePlus™ software v2.2.2 (Applied Biosystems). We used melting curve analysis software of Applied Biosystem to analyze our results. Amplification plots and Tm values were analyzed to affirm the specificities of the amplicons for Sybr Green -based PCR amplification.

Statistics

We used SPSS 20 to do all statistical analyses. Comparisons were done using Mann-Whitney, Krausakul Wallis, chi-square test, and one-way analysis of variance (ANOVA), as appropriate. To investigate the predictive value of chosen RNA based biomarker for BC, we used the receiver operating characteristic (ROC) curve. We assessed the relation between RNA based biomarker expression and clinic-pathological parameters.

P value= level of significance, *P* > 0.05 non-significant, *P* < 0.05 significant, *P* < 0.01 highly significant

RESULTS

The present study included 54 female subjects. They were classified into 3 groups:

Group1: malignant breast cancer cases:

(n=30, of mean age 54.9 ± 14.5 years, median 56 years and range from 20-81 years).

Group 2: benign breast cases diagnosed as fibroadenoma:

(n=12, of mean age 46.5 ± 15.3 years, median 52.5 years and range from 20-62years).

Group 3: healthy normal female individuals:

(n=12, of mean age 50.7± 11.6 years, median 54.5 years, and range from 23-63 years).

Table (1): The Age in Different Groups of the Study in Years.

Group	no.	Median	Range	Mean± SD
Malignant	30	56	20 -81	54.9± 14.5
Benign	12	52.5	20 -62	46.5± 15.3
Healthy normal	12	54.5	23 -63	50.7± 11.6

Table (2): Quantitative RT-PCR for Measurement of lncRNA-HAGLR Mean Rank of Sera Samples in the Malignant Group Compared to Benign and Normal Control Groups.

Group	Quantitative lncRNA-HAGLR by RT PCR
Malignant	
Median	0.4550
Range	0.05 - 4.14
Mean Ranks	18.15
Benign	
Median	36.95
Range	0.34 - 224.41
Mean Ranks	37.75
Normal control	
Median	4.91
Range	0.85 – 265
Mean Ranks	40.63
X ²	24.053
P	0.000**

Kruskal Wallis Test, p (>0.05): not significant, *p (<0.05): significant, **p (< 0.01): highly significant

Using real-time PCR; the mean rank level for lncRNA-HAGLR RNA in the malignant group (Mean rank was 18.15) as compared to benign (Mean rank was 37.75) and normal control groups (Mean rank was 40.63) with high significant difference between the three groups (P < 0.01) (Table 2).

Table (3): Quantitative RT-PCR for lncRNA-HAGLR Comparison between Different Groups of the Study.

Quantitative lncRNA-HAGLR by RT PCR	Malignant and benign groups		Malignant and normal control groups		Benign and normal control groups	
	Malignant	Benign	Malignant	Normal control	Benign	Normal control
Mean rank for lncRNA-HAGLR	17.05	32.63	16.60	33.75	11.63	13.38
χ ² : (P)	46.5 P= (0.000)* *		33 P= (0.000) **		61.5 P= (0.544)	

Mann-Whitney test, p (>0.05): not significant, *p (<0.05): significant, **p (< 0.01): highly significant

By applying post hoc test for lncRNA-HAGLR, there was a highly significant difference between the malignant and benign groups (p <0.01) by using Mann-Whitney test (U=46.5). Also there

was a highly significant difference between the malignant and normal control groups (p <0.01) and (U=33).

But there was no significant difference between the benign and normal control groups (P= 0.544) using Mann-Whitney test (U=61.5)(table 3).

Table (4): lncRNA-HAGLR Expression in Sera Samples in Relation to Clinicopathological Factors of Malignant Group (No=30).

Clinicopathological factors		lncRNA-HAGLR expression		
		Positive(22)	Negative(8)	χ ² :(P)
Parity (30)	Nullipara (4)	3 (13.6%)	1 (12.5%)	0.007 P= (0.935)
	Multipara (26)	19 (86.4%)	7 (87.5%)	
Menopause (30)	Premenopause (9)	5 (22.7%)	4 (50.0%)	2.078 P= (0.149)
	Postmenopause (21)	17 (77.3%)	4 (50.0%)	
Family history (30)	Positive (13)	9 (40.9%)	4 (50.0%)	0.197 P= (0.657)
	Negative (17)	13 (59.1%)	4 (50.0%)	
BMI (30)	Normal (5)	5 (22.7%)	0 (0.0%)	2.240 P= (0.326)
	Overweigh (7)	5 (22.7%)	2 (25.0%)	
	Obese (18)	12 (54.5%)	6 (75.0%)	
OCT (30)	Past administration (20)	15 (68.2%)	5 (62.5%)	0.085 P= (0.770)
	Never (10)	7 (31.8%)	3 (37.5%)	
HT (30)	Past administration (19)	13 (59.1%)	6 (75.0%)	0.639 P= (0.424)
	Never (11)	9 (40.9%)	2 (25.0%)	
Type (30)	IDC (20)	14 (63.6%)	6 (75.0%)	1.705 P= (0.426)
	Mixed (6)	4 (18.2%)	2 (25.0%)	
	Others (4)	4 (18.2%)	0 (0.0%)	
Stage (30)	Stage(I) (12)	10 (45.5%)	2 (25.0%)	1.180 P= (0.554)
	Stage(II) (13)	9 (40.9%)	4 (50.0%)	
	Stage(III) (5)	3 (13.6%)	2 (25.0%)	
Grade (30)	Grade 1 (8)	7 (31.8%)	1 (12.5%)	2.259 P= (0.323)
	Grade 2 (20)	13 (59.1%)	7 (87.5%)	
	Grade 3 (2)	2 (9.1%)	0 (0.0%)	
ER (30)	Positive (16)	11 (50.0%)	5 (62.5%)	0.368 P= (0.544)
	Negative (14)	11 (50.0%)	3 (37.5%)	
PR (30)	Positive (17)	11 (50.0%)	6 (75.0%)	1.493 P= (0.222)
	Negative (13)	11 (50.0%)	2 (25.0%)	
Her2/neu (30)	Positive (7)	6 (27.3%)	1 (12.5%)	0.716 P= (0.398)
	Negative (23)	16 (72.7%)	7 (87.5%)	
subtype (30)	Luminal A (11)	7 (31.8%)	4 (50.0%)	4.839 P= (0.184)
	Luminal B (8)	5 (22.7%)	3 (37.5%)	
	Basal (9)	9 (40.9%)	0 (0.0%)	
	Her2/neu expression (2)	1 (4.5%)	1 (12.5%)	

X²: Chi Square test, p (>0.05): not significant, *p (<0.05): significant, **p (< 0.01): highly significant.

The positivity rate of lncRNA-HAGLR in sera samples was estimated among the malignant group of the study.

The positivity rate of lncRNA-HAGLR was apparently found to be higher in multipara constituting (86.4%) compared to (13.6%) for nullipara. Postmenopausal women constituted (77.3%) in relation to (22.7%) for premenopausal women.

Studying positivity rate and family history showed that (59.1%) of those expressing lncRNA-HAGLR had negative family history compared to (40.9%) for those with positive family history. In malignant cases percent of normal BMI was (22.7%) compared to (22.7%) for overweight and (54.5%) for obese women.

Results also shows that (68.2 %) and (59.1 %) were using OCT and HT respectively in the past, while (31.8%) and (40.9 %) had never used

OCT and HT respectively. (63.6%) of malignant group were IDC compared to (18.2%) for mixed IDC and ILC while other types constitute (18.2%) of lncRNA-HAGLR positive cases.

It was found that (45.5%) of patients were at stage (I), (40.9%) were stage II and (13.6%) were stage (III), While tumors with grade 3 constituted (9.1%) compared to (31.8%) for grade I and (59.1%) for grade 2.

The results also shows that lncRNA-HAGLR positive cases with negative ER & PR represented (50%) compared to (50%) for those with positive ER & PR. The study also shows that samples with negative Her2/neu represented (72.7%) of samples compared to (27.3%) for those with positive Her2/neu.

The correlation between expression of lncRNA-HAGLR and different molecular subtypes of breast cancer was (31.8%), (22.7%), (40.9%) and (4.5%) with Luminal A, Luminal B, Basal and Her2/neu expression Subtype respectively.

No significant correlation was found between lncRNA-HAGLR expression and any of the investigated clinic-pathological factors ($P > 0.05$) (Table 4).

DISCUSSION

Breast cancer (BC) is the most common cancer among women worldwide⁽¹⁾, with an estimated 2.4 million new cancer cases diagnosed in 2015⁽¹⁵⁾. In United States, an estimated 252,710 new cases of invasive breast cancer and 63,410 new cases of in situ breast carcinoma are diagnosed among women in 2017⁽¹⁾. In Egypt, breast cancer accounts for 32 % of total malignancies among females⁽¹⁶⁾. It also occupies the second rank of total cancer cases (18.3%)⁽¹⁷⁾.

Discovering new biomarkers has been the subject of intense research especially with emergence of novel technologies. Major advances in cancer control will be greatly used in early cancer detection⁽¹⁸⁾. With the need for finding biomarkers for early detection of cancer to improve prognosis and survival⁽¹⁹⁾.

Thus the task of bioinformatics in the cancer biomarker discovery is to provide prioritized lists of marker candidates aided by availability of microarrays and bisulfite sequencing and it is anticipated that those key biomarkers identified will

both represent early disease and lead to improved understanding of tumorigenesis⁽²⁰⁾.

Breast CSCs appear to be resistant to hypoxia, chemotherapy and radiotherapy. Moreover, breast CSCs show high tumorigenicity and invasiveness, which are important to the occurrence, development, metastasis and breast cancer recurrence⁽²¹⁾.

Transcription factors such as OCT4, SOX2, NANOG, c-MYC and KLF4, and signaling pathways including Hedgehog, WNT, Notch, PDGF, TGF- β and JAK/STAT, play important roles in sustaining self-renewal capacity in CSCs and therefore provide potential targets in the emergence of therapeutic strategies⁽²²⁾.

In recent years, numerous studies revealed that different lncRNAs could regulate CSCs in many types of cancer via various molecular mechanisms, which include proliferation, differentiation and self-renewal, promotion of metastasis, invasion and expecting prognosis and targeted therapies. Up to date, HOTAIR, H19, ARSR and UCA1 are the most prominent lncRNAs in CSCs. the dysregulation of lncRNAs is a potential biomarker in diagnosis, prognosis, and target therapy of cancers⁽²³⁾.

The lncRNAs are group of non-coding RNAs that regulate gene expression transcriptionally and post-transcriptionally⁽²⁴⁾. The lncRNAs are longer than 200 nucleotides and involved in the pathology of many diseases including cancers⁽²⁵⁾. They became a subject of interest because of thier role in dysregulation in multiple types of human cancers and also they can act as prognostic markers and can be therapeutic targets⁽²⁶⁾.

HAGLR RNA contains eight exons and its transcript is a novel lncRNA. It is transcribed from the HOXD cluster on human chromosome 2q31.2 in the anti-sense manner. HOXD gene is a member of the HOX cluster which regulates organogenesis and embryogenesis. HOX gene dysregulation occurs in multiple types of cancers⁽²⁷⁾. The HOX genes are the key developmental regulators in many processes, involving apoptosis, differentiation and receptor signaling. Dysregulation of HOX genes is frequently implicated in malignancy and plays essential roles in oncogenesis and/or tumor suppression. LncNA-HAGLR is included in regulating the JAK2/STAT3 signaling pathway⁽²⁸⁾.

The results of our study revealed that lncRNA-HAGLR expression was down regulated in breast cancer patients (Mean rank was 18.15) when compared

to benign breast lesions (Mean rank was 37.75) and normal healthy control individuals (Mean rank was 40.63) where there was a highly significant difference among the three study groups as regards fold change (RQ) of serum lncRNA-HAGLR expression ($P < 0.01$).

There was a highly significant difference between the malignant group and benign group ($p < 0.01$) by using Mann-Whitney test ($U=46.5$), also there was a highly significant difference between the malignant group and normal control group ($U=33$) and ($p < 0.01$).

Interestingly, the ROC curve for the lncRNA-HAGLR gene expression in results of our study revealed a cut off value of ≤ 1.045 to discriminate malignant from non malignant cases with a high statistical significance ($p < 0.001$) where an expression level below the cutoff value is considered to be positive because lncRNA-HAGLR expression is down regulated. Using this cutoff value, 22 out of 30 malignant patients were positive ($<$ cutoff value 1.045) for lncRNA-HAGLR expression (73.3%), 1 out of 12 benign patients were positive (8.3%) and 1 out of 12 normal individuals were positive for lncRNA-HAGLR expression (8.3%) at ($P < 0.01$).

Thus lncRNA-HAGLR expression could be used as a sensitive biomarker for early diagnosis of BC with recorded sensitivity 73.3%, specificity 91.6%, PPV 91.6%, NPV 73.3% and accuracy 81.4%.

CONCLUSION

lncRNA-HAGLR detected by quantitative RT-PCR could be used as promising biomarkers for breast cancer.

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