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The Role of Long Non-coding RNA (NKILA and LINC00993) as Tumor Biomarkers in Breast Cancer

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ABSTRACT

Background: Long non-coding RNAs (NKILA and LINC00993) are downregulated in breast cancer (BC) and can have potential use as a novel tumor biomarker. The aim of this work was to investigate the LncRNAs (NKILA and LINC00993) and cytokines (NF-κB and CXCL-1) as potential biomarkers in BC.

Methods: This cross-sectional study included sixty-four pairs of surgically resected human breast cancer tissues and adjacent breast tissues. Expressions of LncRNAs (NKILA, LINC00993) and (NF-κB, CXCL1) cytokines were detected using real-time quantitative polymerase chain reaction (qPCR) analysis,

Results: There was a significant decrease in LncRNAs (NKILA, LINC00993) levels in tumor tissue compared to normal tissue ($P<0.001$). Also, there was a significant increase in NF-κB and CXCL1 levels in tumor tissue compared to normal tissue ($P<0.001$). ROC curve analysis indicated that the LncRNAs (NKILA, LINC00993) expression levels could be considered a promising marker for the diagnosis of breast cancer patients with a sensitivity of 90.6%, 92.2%, respectively. Also, cytokines (NF-κB and CXCL-1) expression levels could be considered a promising marker for the diagnosis of breast cancer patients with a sensitivity and specificity of 87.5%, and 89.1% respectively.

Conclusion: These findings suggest that LncRNAs (NKILA, LINC00993) and cytokines (NF-κB and CXCL-1) can be used as novel biomarkers for breast cancer.

INTRODUCTION

Breast cancer has become one of the most common malignant tumor, and the incidence rate is increasing every year.[1] The incidence is significantly higher in developed countries than in developing countries.[2] In Egypt, BC is estimated to be the most common cancer among females (37.7%) and the leading cause of death (29.1%). The mechanism of breast carcinogenesis is still not fully understood.[3] Early diagnosis of cancer remains a challenge for clinicians; it is an important goal to reduce treatment-associated morbidity and mortality and to reach maximal long-term survival.[4]

Long non-coding RNAs (lncRNAs), which are RNA transcripts with a length greater than 200 nucleotides, have exhibited oncogenic or tumour suppressive roles in the pathogenesis of breast cancer.[5,6] NF-κB Interacting LncRNA (NKILA) can be involved in the pathogenesis of a wide spectrum of human disorders. Numerous studies in hepatocellular carcinoma, breast cancer, melanoma, glioma, and other types of neoplasms have indicated the role of NKILA in the blockage of tumor growth and inhibition of metastasis.[7]

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LINC00993 (Long intergenic non-protein coding RNA 00993) is located on chromosome 10, considerably downregulated in Triple Negative Breast Cancer (TNBC), and associated with Estrogen Receptor (ER) expression.[8] To the best of our knowledge, the precise relationship between NKILA and LINC00993 and BC remains poorly understood.

NKILA has inhibitory roles on NF-κB which belongs to a family of transcription factors that regulate the expression of several molecules and cytokines as C-X-C motif chemokine ligand 1 (CXCL1) participating in various crucial physiological reactions including immune responses, cell proliferation, and differentiation, as well as cell death.[9]

This work aims to investigate the lncRNA (NKILA and LINC00993) and cytokines (NF-κB and CXCL-1) as potential biomarkers in BC.

METHODS

Study design and participants

In this cross-sectional design study, we included sixty-four patients with primary breast cancer who attended the General Surgery Department, at Zagazig University Hospital for partial/total mastectomy. Tumor, nodes, and metastasis (TNM) staging was performed according to the 7th edition of the UICC[10].

Sixty-four tissue samples obtained from tumor tissues represented group A, and sixty-four tissue samples from their normal adjacent tissues served as the control group B.

This study was approved by the Ethical Committee of the Faculty of Medicine, Zagazig University, and following the Helsinki Declaration of 2013. All of the patients were subjected to full history taking and a complete general examination. The diagnosis of all patients was pathologically confirmed after positive mammography. Informed written consent from each participant was taken before participation in the research.

Inclusion criteria

histopathological confirmation of the diagnosis of breast cancer, adequate hepatic, renal, cardiac and respiratory functions.

Exclusion criteria

Male patients, patients with tumors other than breast cancer, those with tumors of unknown origin, patients with histopathological diagnosis other than breast carcinoma, and those who refused to give consent and cooperate.

Tissue sampling and homogenization

The sixty-four pairs of surgically resected breast tissues were immediately frozen and kept at −80 °C. Homogenization of tissue samples was performed using a power homogenizer. Tissue samples were homogenized in about 700µl of GENEzol™ Reagent per 50 mg of tissue using mortar and pestle with liquid nitrogen.

RNA extraction, and reverse-transcription

To quantify gene expression, first RNA was isolated from tissue homogenate using the TriRNA Pure Kit, (Geneaid, China). Complementary DNA (cDNA) was reverse transcribed using a Maxime RT PreMix kit. The cDNA was reverse transcribed in a 20 µL mixture containing 10 µL of total RNA and 10 µL of distilled water. The mixture was incubated at 75ºC for 5 min, then 60ºC for 60 min, 95 ºC for 5 min (RTase inactivation), and ice bath for 5 min.

Quantification of gene expression

The real-time PCR reaction was performed in a total volume of 20 µL containing 5 µL of the cDNA, 10 pmol/µL of each primer (0.5 µL each), 10 µL of TopReal SYBER Green master mix (Enzynomics, Korea), and 8 µL PCR-grade water. The oligonucleotide primers were synthesized by Sangon Biotech (Beijing, China). The primer sequences used for qRT-PCR are shown in Table 1. The real-time RT-PCR was performed in a Rotor-Gene Q 2 plex Real-Time PCR System (Qiagen, Germany). The PCR cycling conditions included an initial denaturation at 95°C for 12 minutes followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The relative expression was calculated with the $2^{-\Delta\Delta ct}$ method.[11]

Table 1. The primer sequences used for qRT-PCR

Name	Forward Primer	Reverse Primer
NKILA	CTTTGGAGGAGTCCAAGCGT	GTGGCTCCAAGAGTGAGCTT
LINC00993	GGCCAAGCACATCTGCAAAA	CCACTGCTTTTCCCAGGACT
NF-KB	GGGGATGGTGAGAAGGTTGG	GCAGTGCCATCTGTGGTTGA
CXCL1	TGGCTTAGAACAAAGGGGCT	AAGGTAGCCCTTGTTTCCCC
Gapdh	CCATGGGGAAGGTGAAGGTC	CTTCCCGTTCTCAGCCATGT

NKILA, NF-kB interacting long noncoding RNA; LINC00993, Long intergenic non-protein coding RNA 00993; NF-KB, Nuclear Factor kappa –light- chain- enhancer of activated B cells; CXCL1, C-X-C motif chemokine ligand 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analysis

Data were analyzed using SPSS version 22, and expressed as mean ± SD for the quantitative parametric variable, and the median for the non-parametric one, with categorical variables expressed as frequency and percentage. Paired Student's t-test was used to compare the two groups in case of normally distributed data, and Mann-Whitney was used to compare them regarding non-normally distributed data when appropriate. We performed Spearman's correlation coefficient test on LINC00993, NF-κB, and NKILA to see if NKILA and LINC00993 influence cancer-related pathways or mechanisms in BC. A P-value of < 0.05 was considered statistically significant. The analysis was based on the accuracy of the identified LncRNA (NKILA and LINC00993) and cytokines (NF-κB and CXCL-1) as a potential biomarker in breast cancer using receiver operator characteristic (ROC) curves as the area under the curve (AUC) value, and sensitivity and specificity.

RESULTS

The age of the studied participants ranged from 40 to 66 years with a mean of 52.75 years. Overall, 57.8% of the patients had a positive family history.

There was a significant decrease in LncRNA (NKILA and LINC00993) in tumour tissue compared to normal tissue (P<0.001). There was a significant increase in NF-κB and CXCL-1 in tumor tissues compared to normal tissue (P<0.001) (Table 2).

Table 2. Expression levels of Long non-coding RNA (NKILA- LINC00993) NF-KB and CXCL-1 among primary breast cancer patients

Variable	Tumor tissue (n=64)	Normal tissue (n=64)	P P
NKILA	0.32±0.22	0.97±0.22	<0.001 <0.001
LINC00993	0.51±0.19	0.97±0.14	<0.001 <0.001
NF-KB	5.47±2.38	1.22±0.66	<0.001 <0.001
CXCL-1	5.43±2.61	1.25±0.69	<0.001 <0.001

NKILA, NF-KB interacting long noncoding RNA; LINC00993, Long intergenic non-protein coding RNA 00993; NF-KB Nuclear Factor kappa –light- chain- enhancer of activated B cells; CXCL1, C-X-C motif chemokine ligand 1.

There was a significant decrease in NKILA in cases with positive family history, Grade 3, negative ER and PR, and N3 compared to other cases (P<0.001). Also, there was a significant decrease in LINC00993 in cases with Grade 3 and N3 compared to other cases (P<0.001). There was a significant increase in NF-κB in cases with negative Er, PR, and N3 compared to other cases. Also, there was a significant increase in CXCL-1 in cases with tumour size >5, ILC type, grade 3, and T3 or 4 compared to other cases (Table 3). There was a positive correlation between LINC00993, NF-κB, and NKILA (Table 4).

Table 3. Relation between family history, clinicopathological parameters, and studied parameters among primary breast cancer patient:

		No	%	NKILA	P	LINC00993	P	NF-κB	p	CXCL-1	P
Family history	-ve	27	42.2	0.42	<0.001	0.52	0.19	5.6	0.96	5.82	0.59
	+ve	37	57.8	0.22**		0.47		5.43		4.86	
Tumor size	< 5	39	60.9	0.28	0.12	0.49	0.71	5.54	0.44	4.17	0.003
	≥ 5	25	39.1	0.22		0.47		5.60		6.59*	
Tumor type	IDC	55	85.9	0.28	0.002	0.50	0.01	5.60	0.38	4.86	0.003
	ILC	9	14.1	0.12*		0.34*		4.69		7.42*	
Tumor grade	1 or 2	47	73.4	0.30	<0.001	0.51	<0.001	5.78	0.39	4.86	0.008
	3	17	26.6	0.12**		0.34**		4.69		6.73*	
T	T2	37	57.8	0.28	0.45	0.49	0.90	5.54	0.67	4.57	0.03
	T3 or 4	27	42.2	0.22		0.47		5.60		6.59*	
ER, PR	-ve	53	82.8	0.23	<0.001	0.49	0.64	5.82	0.04	5.03	0.73
	+ve	11	17.2	0.75**		0.41		5.43*		5.82	
N	N1	9	14.1	0.79	<0.001	0.52	<0.001	4.2	0.03	5.82	0.06
	N2	35	54.7	0.28		0.36		5.54		4.57	
	N3	20	31.3	0.12**		0.34		6.54*		6.73	

MW: Mann Whitney; KW: Kruskal Wallis test
*: Significant (P<0.05)
**: highly significant (P <0.001)

NKILA, NF-KB interacting long noncoding RNA; LINC00993, Long intergenic non-protein coding RNA 00993; NF-KB, Nuclear Factor kappa –light- chain- enhancer of activated B cells; CXCL1, C-X-C motif chemokine ligand 1. Tumors we regraded according to the modified Bloom–Richardson system; T size of the tumor, T2 (2–5 cm), T3 (>5 cm), T4 (infiltration of the chest wall/skin), N regional lymph node involvement, N1 cancer has spread to one to three axillary lymph node(s), and/or tiny amounts of cancer are found in internal mammary lymph nodes on lymph node biopsy, N2 cancer has spread to four to nine axillary lymph nodes, or cancer has enlarged the internal mammary lymph nodes, N3 cancer has spread to axillary lymph nodes, the internal mammary lymph nodes and/or infraclavicular and supraclavicular lymph nodes; ER and PR, estrogen receptor and progesterone receptor.

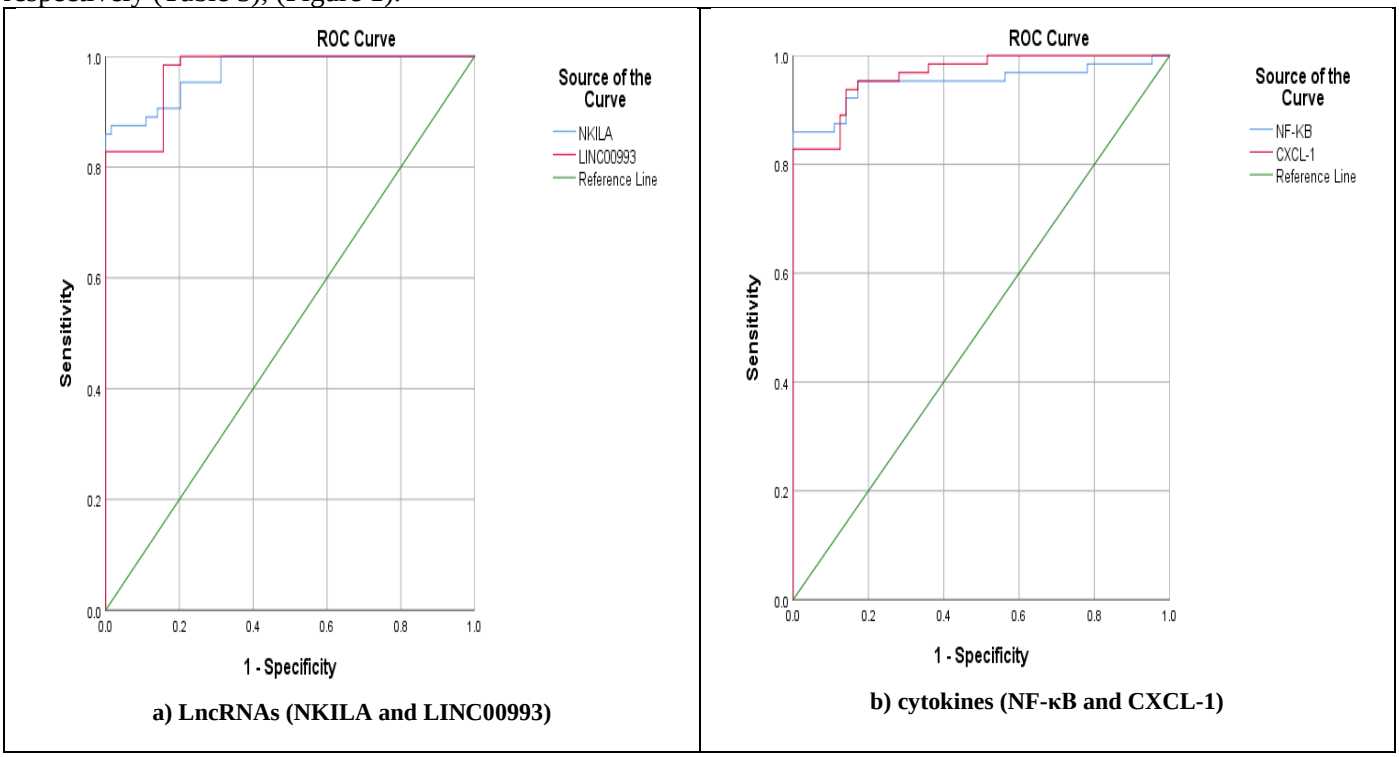
Long non-coding RNA (NKILA, LINC00993) and cytokines (NF-κB, CXCL-1) as potential biomarkers in breast cancer

The sensitivity and specificity of the studied parameters' expression levels as biomarkers in BC were assessed in the breast cancer tissues using ROC curve analysis. ROC curve analysis indicated that long non-coding RNA (NKILA, LINC00993) expression levels could be considered a diagnostic parameter for BC with a sensitivity of 90.6%, 92.2%, respectively and specificity of 85.9 % and 84.4 %, respectively. Cytokines (NF-κB, CXCL-1) expression levels could be considered a diagnostic parameter for breast cancer with a sensitivity (87.5%, 89.1% respectively) and specificity of 89.1 %, 87.5 % respectively (Table 5), (Figure 1).

Table 4. Correlation between Long non-coding RNA, NF-KB, and CXCL-1 among primary breast cancer patients

	NKILA r	LINC00993	NF-KB
LINC00993	0.63**		
NF-KB	-0.29*	0.04	
CXCL-1	-0.10	-0.23	0.10

r: Spearman's correlation coefficient.
*: Significant (P<0.05) **: highly significant (P <0.001)
NKILA, NF-KB interacting long noncoding RNA; LINC00993, Long intergenic non-protein coding RNA 00993; NF-KB, Nuclear Factor kappa –light- chain- enhancer of activated B cells; CXCL1, C-X-C motif chemokine ligand 1.



a) LncRNAs (NKILA and LINC00993) **b) cytokines (NF-κB and CXCL-1)**

Figure 1. Roc curve for validity of a) LncRNAs (NKILA and LINC00993) and b) cytokines (NF-κB and CXCL-1) in diagnosis of tumor among the studied cases

DISCUSSION

Breast cancer is the most common cancer in women and represents the leading cause of cancer mortality among this gender worldwide. It stands as the most frequent malignancy in females, thus signifying a principal focus of biomedical research.[12]

The prevalence and incidence rates of BC are progressively escalating across the world. The National Cancer Registry Program's 2013 report in Egypt reveals that breast cancer is the most pervasive form of cancer among females, accounting for 32% of all female cancer cases.[13,14]

Table 5. Validity of studied parameter in the diagnosis of tumor among the studied cases:

	Cut off	AUC(CI 95%)	Sensitivity	Specificity	PPV	NPV	Accuracy	p
NKILA	<0.75	0.97(0.94-1)	90.6%	85.9%	86.6%	90.1%	87.5%	<0.001
LINC00993	<0.88	0.97(0.95-0.99)	92.2%	84.4%	85.5%	91.5%	88.3%	<0.001
NF-KB	>2.05	0.95(0.91-0.99)	87.5%	89.1%	88.9%	87.7%	85.9%	<0.001
CXCL-1	>1.16	0.96(0.93-0.99)	89.1%	87.5%	87.7%	88.9%	85.9%	<0.001

AUC: Area under curve, CI: Confidence interval, PPV:+ve predicted value, NPV:-ve predicted value, NKILA, NF-KB interacting long noncoding RNA; LINC00993, Long intergenic non-protein coding RNA 00993; NFκB, Nuclear Factor kappa –light- chain- enhancer of activated B cells; CXCL1, C-X-C motif chemokine ligand 1.

Innovative biomarkers are required for early detection, treatment, and prognosis. Non-coding RNAs (ncRNAs) have emerged as crucial players in various stages of breast cancer tumorigenesis, influencing cell death, metabolism, epithelial-mesenchymal transition (EMT), metastasis, and drug resistance.[15]

The current study showed a significant decrease in expression levels of lncRNA (NKILA) in tumor tissues compared to normal ones (P < 0.001). NKILA expression levels in tissues showed high sensitivity and specificity, and, therefore, had a significant diagnostic value for BC patients.

Consistent with our results, studies by Di Huang *et al.*, Li-Hua Luo *et al.* also reported a decrease in NKILA expression in BC tissues and its involvement in inhibiting tumor progression, including cell proliferation, migration, and angiogenesis, indicating its potential as a therapeutic target.[16,17] Our findings align with those of several other studies on various cancer types, such as non-small cell lung cancer, melanoma, hepatocellular carcinoma (HCC), and rectal cancer, where NKILA expression was also found to be downregulated in tumor tissues.[18,21]

The current study found that altered NKILA expression was associated with specific tumor characteristics in BC, including positive family history, invasive lobular carcinoma, high-grade tumors, negative estrogen, progesterone receptors, and advanced lymph node involvement. These findings suggest that NKILA could serve as an important biomarker in BC diagnosis and prognosis.

Consistent with our results, Huang and colleagues found that NKILA levels decreased in breast cancerous tissue compared to non-cancerous tissues and were correlated with lymph node involvement and higher TNM stage.[16] Moreover, studies by Luo and Wu reported correlations between NKILA expression and clinical stage, TNM classification, and EMT features.[17,22] Liu *et al.* found that NKILA expression was an independent prognostic factor. Furthermore, low NKILA expression is associated with breast cancer metastasis and poor patient prognosis which confirms our results.[23]

NF-κB significantly increased in tumor tissues compared to normal ones (P<0.001) and a significant negative correlation was found between NKILA and NF-κB in breast cancer. This suggests a regulatory interplay between these molecules, where higher NKILA expression is associated with lower NF-κB expression and vice versa. This inverse correlation may indicate that NKILA has a potential inhibitory role in suppressing NF-κB activity, which plays a critical role in cancer-related processes, thereby affecting BC pathogenesis. In accordance with our findings, Huang *et al.*, have demonstrated that NKILA interacts with the NF-κB –IκBα complex by binding to p65 and modulates T cell activation-induced cell death by inhibiting NF-κB activity.[16]

The current study has demonstrated an increase in CXCL-1 expression in BC tissue as compared to normal tissue (P<0.001). This elevation was particularly prominent in cases characterized by tumor sizes exceeding 5, invasive lobular carcinoma (ILC) type, cases graded as 3, and those with tumor stages classified as T3 or T4. These findings underscore the upregulation of CXCL-1 in breast cancer, specifically in aggressive subtypes.

These findings are in line with those reported by Wang et al., who found a correlation between elevated CXCL1 expression and advanced cancer stage, lymph node metastasis, and poor survival. The findings highlight the value of the CXCL1- NF-κB axis in stimulating BC growth and metastasis.[24]

The results showed that CXCL1 and NF-κB may serve as a potential therapeutic target for the prevention of cancer growth metastasis.

The current study found a highly significant decrease in LINC00993 expression in breast cancer

tumor tissues compared to normal tissues ($P < 0.001$). LINC00993 expression levels showed high sensitivity and specificity, and thus have a significant diagnostic value for BC patients.

In accordance with our results, some studies reported significant downregulation of LINC00993 in TNBC and its association with luminal BC.[8,25,26]

The study by Guo *et al.* found that higher expression of LINC00993 was associated with a better outcome in TNBC patients, indicating its prognostic value.[8]

Our study found that the expression of LINC00993 was associated with invasive lobular carcinoma (ILC) type, high-grade tumors (Grade 3), and advanced lymph node involvement (N3). These findings suggest that LINC00993 may have a significant role in BC pathogenesis as their expression levels are associated with specific tumor characteristics. Chen *et al.*, demonstrated discernible patterns of LINC00993 expression across various subtypes of BC, thereby underscoring its potential significance in distinguishing between different types of BC. These findings are consistent with the results of our study.[26]

The current study found a positive correlation between the expression levels of NKILA and LINC00993 in cases of BC. This finding suggests a potential for these lncRNAs to regulate the pathogenesis of BC. The observed positive correlation supports the notion that NKILA and LINC00993 may function in tandem to influence cancer-related pathways or mechanisms in BC. These lncRNAs hold promise as tumor biomarkers and therapeutic targets for personalized BC treatment strategies.

CONCLUSION

These findings suggest that LncRNAs (NKILA, LINC00993) and cytokines (NF-κB and CXCL-1) can be used as novel biomarkers for BC.

Our study is a preliminary study in the Egyptian population, showing that LncRNAs (NKILA, LINC00993) and cytokines (NF-κB and CXCL-1) have potential diagnostic value with adequate sensitivity and specificity for BC and could be considered as valuable markers for BC diagnosis. Also, they may be serve as promising prognostic tumor markers, in selecting candidate patients for a more aggressive adjuvant treatment or novel therapeutics options.

Future large-scale studies are needed to confirm our findings and to further explore the existing potential of the studied parameters as novel biomarkers for breast cancer in order to be utilized in the clinical field.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

ETHICAL CONSIDERATIONS

This study was approved by the Ethical Committee of the Faculty of Medicine, Zagazig University, and following the Helsinki Declaration of 2013.

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DATA AVAILABILITY

The corresponding author can be contacted directly for access to the data.

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