Original Article

Expression of Intragenic LCAL4 Long Non-Coding RNAs as a Potential Diagnostic and Prognostic Marker in Female Breast Cancer

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Received November 2023; Revised and accepted March 2024

Abstract

Objective: In breast cancer early detection is associated with reduced mortality and it is essential to identify new biomarkers for early detection and appropriate management of cancer patients with the best response to treatment. Long non-coding RNAs (LncRNAs) have attracted much attention as potential diagnostic, prognostic, or predictive biomarkers due to their high specificity, easy access to non-invasive methods, and their aberrant expression under various pathological and physiological conditions. Have attracted the aim of this study is to investigate the expression profile of intragenic non-coding LncRNAs LCAL4 as a biomarker as potential diagnostic and prognostic biomarkers in cancer.

Materials and methods: In this research, 62 tissue samples were obtained from patients undergoing therapeutic surgery in Khatam al-Anbia Hospital and the normal peripheral tissue that was removed for prevention was used as a control by Real-time PCR method.

Results: The expression pattern of LCAL4 long non-coding RNA gene is significantly different between two groups of healthy control samples and samples obtained from patients with different breast cancer subtypes, Also its expression between samples obtained from different subgroups and different stages showed significant differences.

Conclusion: The studied LncRNAs can act as a factor to identify tumor tissue from healthy tissue, and the diagnosis of cancer grades can be different depending on the type of LncRNA. These results can be proposed in the introduction of LncRNA LCAL4 as a new marker in the diagnosis of breast cancer. In addition, by interpreting the results, it can be concluded that these LncRNAs can be considered as influential factors in the process of breast cancer.

Keywords: Long Non-Coding RNA (LncRNA); Breast Cancer; Cancer Marker

Introduction

Breast cancer is a very heterogeneous disease that is caused by the interaction of genetic risk factors and environmental factors and leads to the gradual accumulation of genetic and epigenetic changes in breast cancer cells. Although epidemiological evidence highlights the existence of risk factors (such as age, obesity, alcohol consumption, and lifetime estrogen exposure), a family history of breast cancer is the strongest risk factor (1, 2).

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Approximately 20% of all breast cancers have a familial origin, and in terms of etiology, they depend on a specific predisposing gene for that disease (3). Cancer is generally a genetic disease, in other words, all cancers, including breast cancer, are caused by gradual genetic changes in tissue cells and the accumulation of these mutations, in such a way that these mutations cause cancer and overgrowth of that cell in several ways which eventually leads to cancer. Histopathological analysis of breast tumors plays an essential role in the diagnosis of breast cancer. For example, invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC) and histologic grade (summary score of epithelial tube formation, mitotic count, and nuclear pleomorphism) have been reported to guide clinical management. Microscopic assessment of tumor-infiltrating lymphocytes can predict improved response to chemotherapy and prognosis in erb-b2 receptor tyrosine kinase (HER2) positive breast cancer. Beyond these features, breast tumors show a set of other morphological features such as necrosis, whose clinical significance is not well defined?

Breast cancer is a heterogeneous disease at both morphological and molecular levels. PAM50 molecular “intrinsic” subtypes, duct A, duct B, HER2-associated, basal-like, and normal-like, have distinct biological characteristics, epidemiological risk factors, treatment response, and prognoses, and are associated with specific morphologic features. The normal-like subtype is highly variable and is not recursively defined. According to the American Joint Committee on Cancer, breast cancer stages can be divided into the TNM system: T: the size of the breast tumor, N: the extent of tumor spread to nearby lymph nodes, and M: the extent of the tumor. Metastasis to other organs of the body is the first stage of breast cancer called stage zero or carcinoma in situ. In the first stage, the tumor is small and has not spread outside the patient's breast. Stage II cancer is less than 2 cm in diameter and may also be seen in some lymph nodes under the armpit. In the third stage, the tumor found in the breast may be of any size, but the armpit cancer will not be equivalent to the second stage. In addition, the cancer has spread to the chest wall or the skin of the breast and has caused indentation, inflammation or discoloration of the skin of the breast. Finally, in stage IV breast cancer, the cancer has spread to distant parts such as the brain, lungs, or bones (4). Most breast cancers are epithelial tumors that arise from the cells lining the ducts or lobules. Non-epithelial cancers of the supporting stroma are less common (such as angiosarcoma, primary stromal sarcoma, phyllodes tumor). According to the presented cancer characteristics, the cancer cell's ability to invade and metastasize is an important factor in determining the aggressive characteristics of the disease and is a promising molecular target for drug discovery (5) (Figure 1).

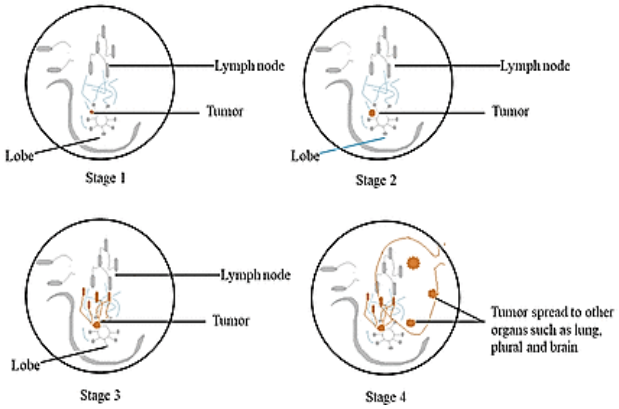

Figure 1: Stages of metastasis in breast cancer (6)

Morphological and molecular data complete the description of breast cancer phenotypes. For example, basal tumors do not show high histologic grade, necrosis, tumor-infiltrating lymphocytes, and fibrotic foci and are generally IDC, whereas HER2-associated tumors show high histologic grade and may It contains features of apocrine and ductal carcinoma in situ (6, 7). Although several genetic factors have been identified as causing cancer, new factors are always added to this list, and recently RNA genes have also been included in this list. which includes micro RNA genes, genes encoding long RNAs, etc. Circular RNAs (circRNAs) were recently discovered as a circular subset of endogenous RNAs with the ability to regulate gene expression by microRNA. The researchers found that a total of 715 circRNAs were significantly overexpressed and 440 were significantly downregulated in breast cancer lesions compared to healthy tissue samples among 1155 differentially expressed circRNAs. In 2019, Yan et al. introduced hsa_circ_0072309 as a new prognostic biomarker, which is a downregulated miR-492. Dysregulation of this circRNA increases the proliferation, migration and invasion of breast cancer cells and therefore has a potential role in breast cancer (8). In breast cancer lesions compared to healthy tissue samples among 1155 differentially expressed circRNAs it was discovered that a total of

715 circRNAs were significantly overexpressed and 440 were significantly downregulated. A novel prognostic biomarker was hsa_circ_0072309 as, which is a downregulated miR-492 (9). Dysregulation of this circRNA increases the proliferation, migration and invasion of breast cancer cells and therefore has a potential role in breast cancer (10). LncRNAs that differ from each other in terms of nucleotide composition can still show the same three-dimensional structure and therefore the same molecular function (11). In several human diseases, including cancer he expression of these non-coding molecules is highly regulated (12 Some LncRNAs can encode micropeptides despite their classification as non-coding molecules. As a current concept any newly investigated LncRNA should be validated as a non-coding transcript before drawing conclusions about its regulatory role (13, 14).

Expression of ncRNAs is not limited to classical mechanisms. A type of genome editing causes the creation of circular RNAs (circRNA). CircRNAs are made of a covalently closed loop and therefore lack a 5' cap and 3' tail. Such RNAs are well conserved, relatively stable and often tissue specific. Many miRNAs are already. LncRNAs and circRNAs are evident to be successfully used as biomarkers or therapeutic targets for many different diseases (15-17). Two main categories of regulatory RNAs: small and long ncRNAs are defined according to length of ncRNAs (18). That are endogenously expressed and regulate gene expression at the post-transcriptional level. Since their first description in the worm Caenorhabditis elegans, thousands of miRNAs have been identified in flies, plants and mammals. The conserved structure and biogenesis of MiRNAs defined as single-stranded ncRNAs with a length of 20 nucleotides affects modulation of gene expression by miRNAs (19). LncRNAs comprise the largest proportion of non-coding transcripts and are of great interest. In general, the term LncRNA refers to transcripts that have more than 200 nucleotides and do not code for proteins and stays the separation cutoff for lncRNAs from smaller ncRNA species such as miRNAs, siRNAs. There is no open reading frames or conserved codons in transcripts for "non-coding" cases (20). In sum, the conservation of LncRNAs should be defined from a different angle considering sequence, structure and function to fully define the evolutionary relationship between LncRNAs of different species (21, 22). The biogenesis of LncRNAs occurs in the nucleus. Unlike mRNAs, LncRNAs can be found in different parts of the cell. After biogenesis and processing, mRNAs are released in the cytoplasm, which is also true for several LncRNAs, while most non-coding transcripts remain in the nucleus and are absorbed into chromatin (Figure 2).

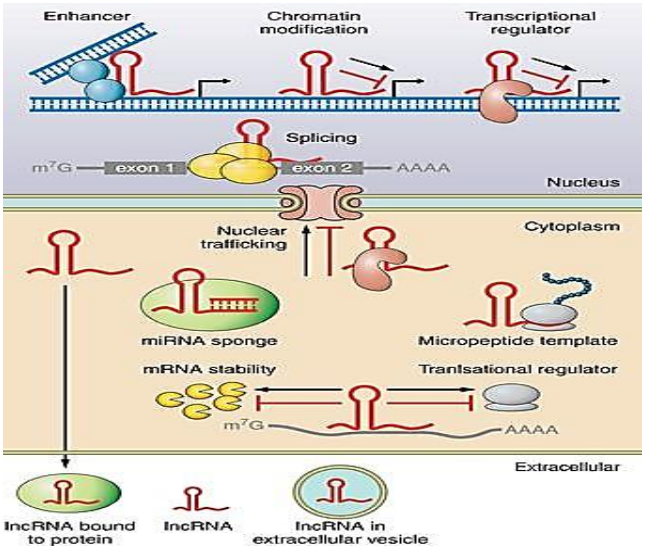

Figure 2: Biogenesis and function of long non-coding RNAs (LncRNAs) (18)

LncRNAs control the expression of genes in the nucleus by interacting with DNA, chromatin modifying complexes or different transcriptional regulators (23). Although LncRNAs are more structured than their protein-coding counterparts, their structural versatility may play an important role, enabling LncRNAs to form binding sites to interact with proteins, DNA, and other RNA molecules (23).

Also, the effect of LncRNAs such as ecCEBPA, MLK7-AS1, TUG1, HOXA11-AS, GAPLINC, LEIGC, and LncRNA associated with multidrug resistance and upregulation, PVT1 on cancer epigenetic and drug resistance as well as their potential as therapeutic targets for personalized medicine. It has been discussed (24). The expression pattern of long-term non-coding RNA (LncRNA) of non-small cell lung cancer (NSCLC) and their potential biological function has been investigated on a genome-wide scale. LCAL4 expression level is related to tumor stage, but has no relationship with age and sex. Considering that LCAL4 is the most aberrantly expressed LncRNA in NSCLC tissues and is also significantly expressed in NSCLC patients from the TCGA database, LCAL4 can be a prognostic indicator and has the potential to be a

diagnostic biomarker of NSCLC (26). In a study aimed at identifying LncRNA with a strong prognostic value for breast cancer LCAL4 was identified as a potential biomarker for predicting BRCA prognosis (27). Investigating the role of long non-coding RNAs in the genetic and epigenetic regulation of the genome is one of the most exciting new aspects in genetics. The role of Lnc-RNA in the processes related to all types of cancer has been proven and many researches have been conducted in this field in different parts of the world, however, in Iran, the number of such studies is very few. The results of this research can be an important step for the diagnosis of the disease, its treatment and management, and the subsequent follow-up of the patients. On the other hand, by clarifying the role of Lnc-RNA and examining the differences in the expression profile of these molecules in patients and in different stages of the disease compared to normal people, they can be used as biomarkers. Also, the results of this study can be used in determining breast cancer diagnostic panels. A total of 1392 LncRNAs with different expression have been identified. LCAL4 was the most aberrantly expressed LncRNA in NSCLC compared to NTL. Upregulation of LCAL4 in NSCLC patients is confirmed from TCGA database (28, 29). Furthermore, in the TCGA database, LCAL4 is significantly upregulated in both squamous cell carcinoma and adenocarcinoma, and high LCAL4 expression is associated with poor overall survival of NSCLC patients (30).

Materials and methods

The type of current study is of fundamental application type. The study population is breast cancer patients who underwent surgery in Khatam al-Nabiya hospital in Tehran in 1401. In this study, 62 samples were prepared from the patients and the marginal normal tissue that was removed for prevention was used as a control. The method of breast tissue biopsy is to extract miRNA. A piece of tissue was removed from the body and examined in the laboratory. For breast biopsy, the breast tissue is removed by a special needle, in some cases it is removed during the surgery to determine whether there is cancer or abnormal cells. Here we used the surgical method. The outcome measurements are summarized in table 1.

In this study, a total of 62 patients from Khatam Al-Anbia Hospital were performed, and a biopsy was prepared by a surgeon from a sample of healthy tissue and tumor tissue and placed in a microtube **.**In order to perform Real Time PCR, a fluorescent reporter molecule was used to observe the progress of PCR and the amount of products produced during a PCR experiment was compared with the amount of products produced during PCR experiments with a certain amount of initiator nucleic acid, and it was possible to find out the amount of nucleic acid. The primary acid in the sample was provided. For molecular investigation of genes RNA extraction was performed according to the Trizol method. A spectrophotometer (Nanodrop) was used to check the amount of extracted RNA. The synthesis of cDNA was done by Pishgam kit as one step and some as two steps. In the two-step mode, cDNA synthesis was first performed in one vial, and then normal PCR was performed to check the gene expression of the synthesized cDNA in another vial. Then we mixed the desired RNA with the above-mentioned ingredients on ice and transferred the vial to the thermocycler to apply the necessary temperatures with the schedule that varies depending on the kit.

Primer design for studied LncRNAs and β-actin gene as an internal control was first extracted and conserved region using the NCBI database of variants and sequences of investigated genes. The design of primers was done using Oligo7 software for the desired variant in such a way that it does not connect to the genome and only identifies the desired variant. The Primer Blast software was also used to check the primers.

Primer checking was performed using GraphPad Prism 9.2.0 software. In order to analyze the data that had a normal distribution, parametric tests were used, and for the data that did not have a normal distribution, non-parametric tests were used.

Table 1: Research variables and their definition

Variable	Functional definition	Unit of measure
Study groups	Witness samples Tumor samples	Cells that grow abnormally due to various factors and eventually cause tumors. Healthy cells and tissues that are considered as control samples
The expression level of the target gene	Fold Change	Measuring the number of copies with Real-Time PCR
The expression level of LCAL4	Fold Change	Measuring the number of copies with Real-Time PCR

To compare continuous variables between two groups, two-sided t-test was used, and to compare three or more groups, one-way ANOVA was used. $P \leq 0.05$ was assumed to be significant.

In order to collect samples and clinical information and to participate in the present study, written consent was obtained and kept from all patients. All the information obtained from the investigated persons in all stages of the research, including the publication of the results, will be confidential.

Results

The diagram and melting curve for the studied gene in breast tumor tissue, normal breast tissue and negative control sample are shown in Figure 1. Beta-actin is the internal control gene that is expressed in the studied sample. LncRNA LCAL4 gene was expressed in tumor and normal breast tissue samples, but it was not expressed in the normal peripheral tissues of the tumor, which was used as a negative control (Figures 3-6).

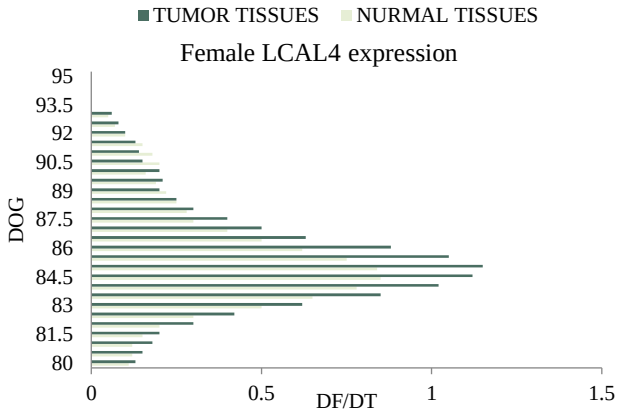

Figure 3: LCAL4 gene fusion diagram

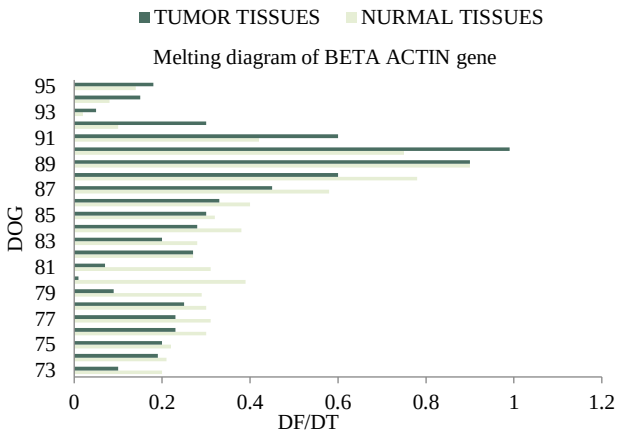

Figure 4: Beta actin gene fusion diagram

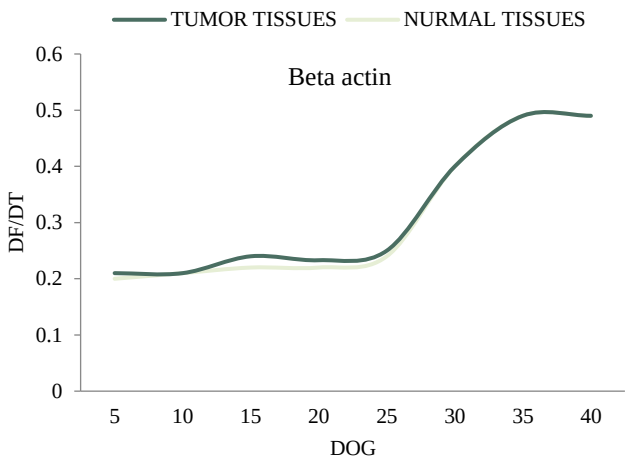

Figure 5: Diagram of Beta Actin gene replication

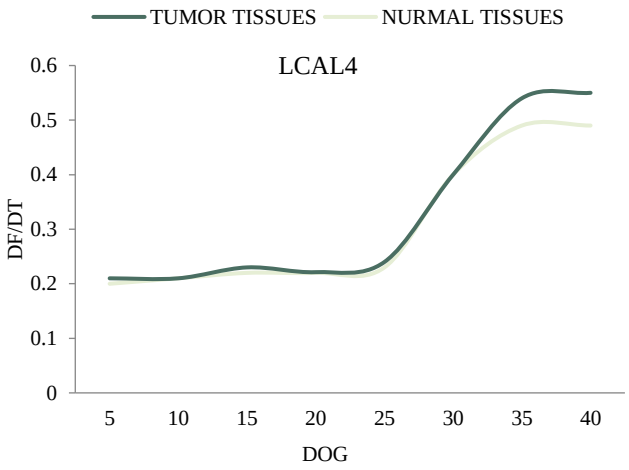

Figure 6: Duplication diagram for LCAL4 gene

In examining the expression of studied genes in the study of 62 studied samples, the expression of LINC in cancer samples was significantly higher than that of normal samples (Figure 7). As can be seen the expression values of LCAL4 LncRNA in the tumor sample compared to the normal tissue sample is several times higher.

Among the studied cancer biopsies, LINC expression was significantly higher in grade 1 and 2 samples than grade 3 samples. In grade 1 and 2 samples LINC expression is several times higher than grade 3 samples (Figure 8).

The expression of LINC in the studied samples was examined in terms of the presence or absence of the estrogen receptor. The results showed that there is no significant difference in the expression of LINC among samples with or without estrogen receptor (Figure 9).

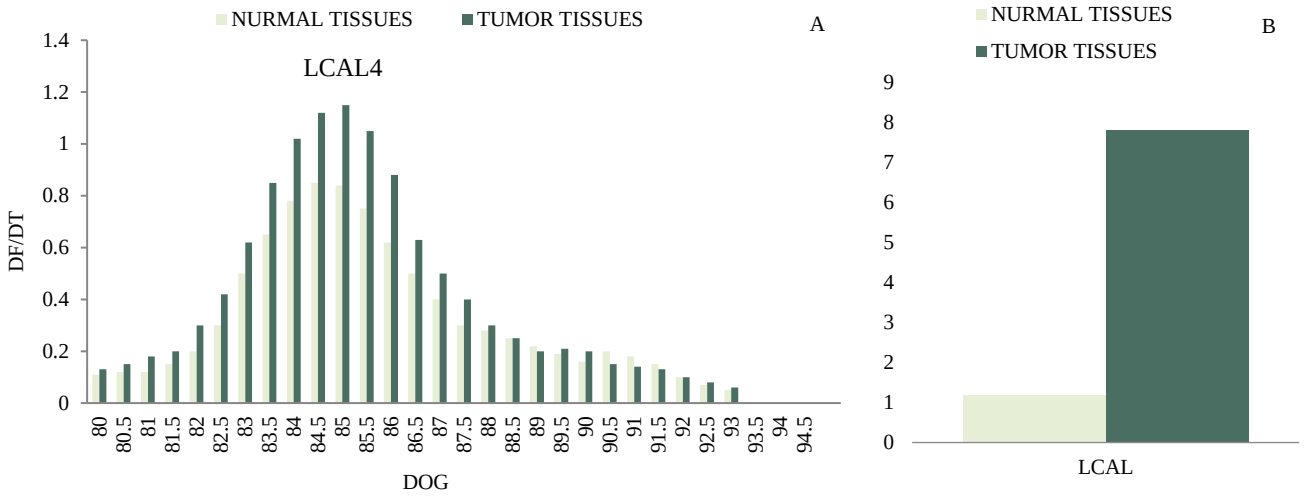

Figure 7: A and B) LCAL4 LncRNA expression between healthy and cancerous tissues (P < 0.0001)

Profiling has led to a new molecular classification of breast cancer, which is characterized by four intrinsic subtype: Triple-negative breast cancer, HER2 positive, LuminalA and Luminal B channel. The expression of LCAL4 LncRNA in the Luminal B subtype was higher than the others, but no significant difference was observed among the other studied subtypes (Figure 10).

Discussion

Comparison of malignant cells with corresponding normal cells has shown that many transcription factors, post-transcriptional regulators such as RNA binding proteins, microRNAs and LncRNAs are critical regulators to promote or inhibit tumor growth. LncRNAs have attracted considerable attention in terms of regulating neoplastic progression as well as in regulating various cellular functions, including proliferation, migration, and DNA stability. On the other hand, due to the specific expression of different RNAs in some cancers, these nucleotide sequences have gained an important role in cancer diagnosis (8). LncRNAs play an important role in a variety of biological processes, regulating the physiological functions of organisms, including epigenetic control of gene regulation, transcription and post-transcription on various aspects of cellular homeostasis including proliferation, survival, migration and genomic stability. LncRNAs are also able to regulate gene expression and influence cellular signaling cascades, playing an important role in promoter-specific gene regulation and X chromosome inactivation.

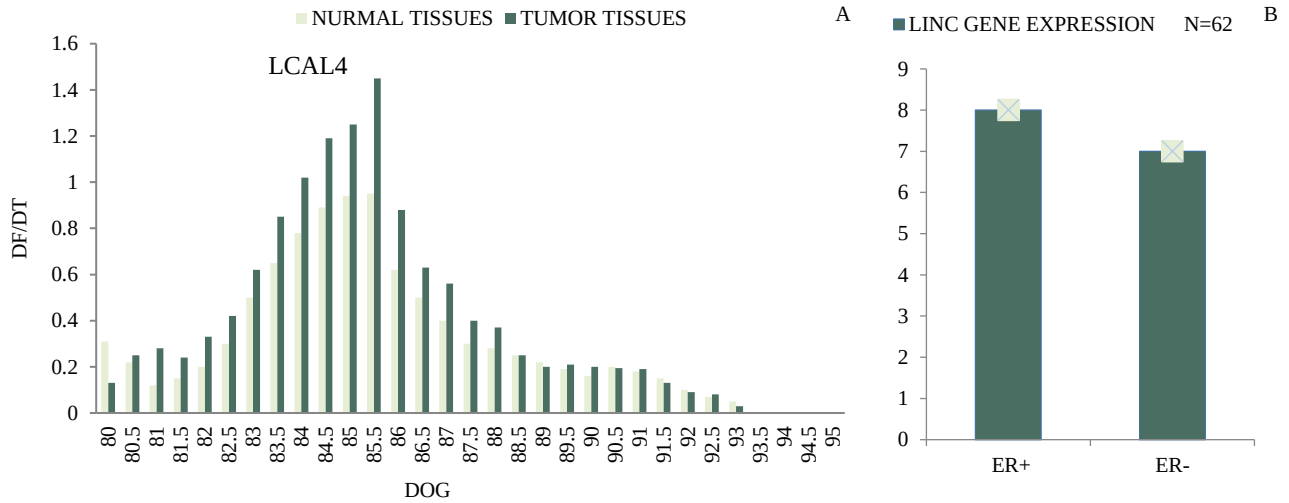

Figure 8: A and B) Expression of LCAL4 LncRNA in different grades of cancer (P < 0.01)

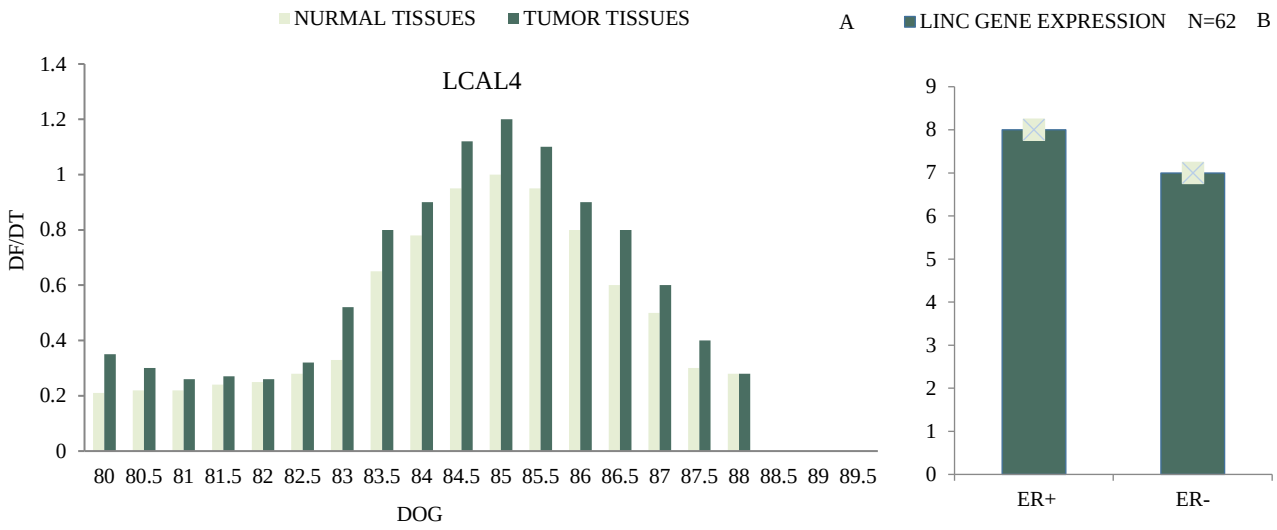

Figure 9: A and B) Expression of LCAL4 LncRNA in biopsies prepared based on estrogen receptor (P < 0.01)

In addition, LncRNAs have been reported to interact with DNA, RNA or protein molecules and regulate chromatin organization, transcriptional and post-transcriptional regulation. Consequently, they are differentially expressed in tumors and are directly related to the transformation of healthy cells into tumor cells.

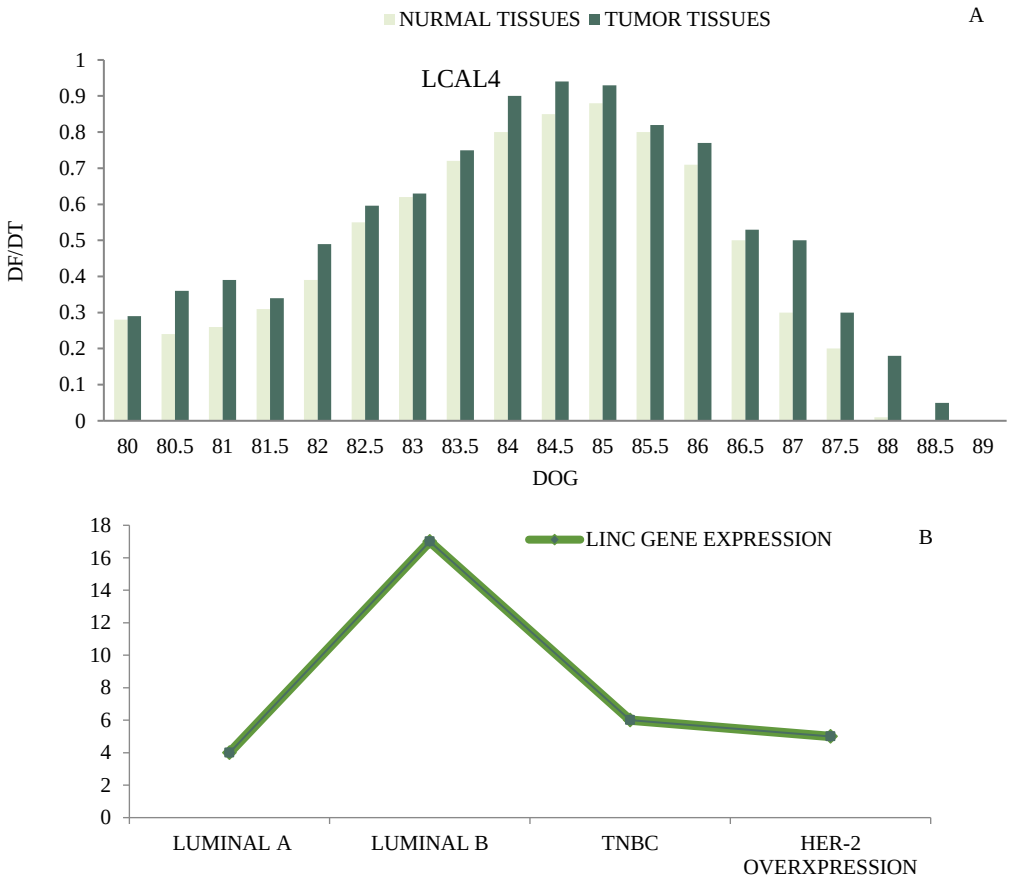

Figure 10: A and B) LCAL4 LncRNA expression in different cancer subtypes (P< 0.01)

As a result of their key functions in a wide variety of biological processes, LncRNAs are becoming emerging agents in biology and medicine, with potential functional roles in various cancers. In this study, it has been shown that the studied LncRNAs have different expression in different grades of breast cancer, so the expression of LncRNA LCAL4 is higher in grade 1 and 2 samples than grade 3.

It has been shown that most women with stage I, II or III breast cancer are treated with surgery, often followed by radiation therapy (Figure 11). Many women also receive some form of systemic drug therapy. In general, the more advanced the breast cancer is, the more likely it is to need treatment. Therefore, understanding the stage of cancer plays an important role in its treatment. According to this point, it can be said that the existence of the factor causing the difference, such as the factors examined in this study, can determine the grading of this cancer and can be used as a molecular criterion in this field (28).

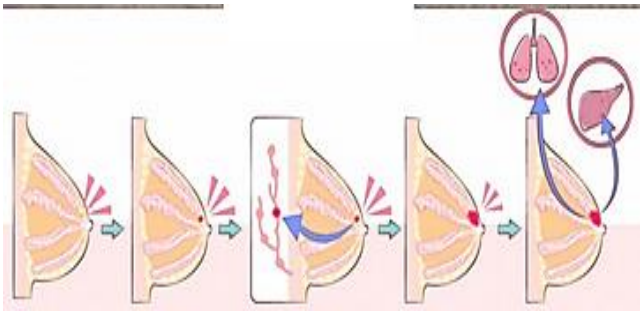

Figure 11: Different stages of breast cancer

Estrogen and progesterone receptors, which are present in some breast cancers, are nuclear hormone receptors that, when the right hormones bind to them, promote DNA replication and cell division. In this study, there was no significant change in the expression of LCAL4 in the samples compared to being estrogen positive or negative. This factor can also increase the diagnostic marker value of this LncRNA. Yuan et al.'s study showed that estrogen receptor alpha (ERα) plays a vital role in the development of normal breast tissue and in breast cancer. By cross-analyzing the Cancer Genome Atlas (TCGA) database, ERα- regulated long non-coding RNA 1 (ERLC1) was identified as a long non-coding RNA showing a strong association with ERα signaling and high expression specificity in breast tissue. ERLC1 is transcriptionally activated by ERα, and ERLC1 enhances ESR1 transcription by sequestering mir-129 and binding to FXR1. ERLC1 is increased in tamoxifen-resistant breast cancer cells, where knockdown of ERLC1 restored sensitivity to tamoxifen and increased the efficacy of palbociclib or folostrant treatment (31). In Zhao et al.'s study, 6 LncRNAs associated with estrogen receptor and breas cancer metastatic grade have been introduced. These 6 LncRNAs including LINC00917, AL391840.1, TRIM52-AS1, AL355075.4, AC093802.2, and AC091544.4, significantly predict survival in patients with grade III and estrogen receptor-positive patients. In addition, low-risk patients treated with tamoxifen had a longer disease-free survival than those who did not receive any treatment. Overall, determination of six LncRNAs can be a potential prognostic tool to predict patients' survival and to predict the benefit of tamoxifen treatment in BRCA. These results show the role of certain LncRNAs in treatment and drug response and justify the results obtained in the present study (32). Therefore, the difference in the expression of a LncRNA can be different in determining the treatment line, depending on the molecular marker of breast cancer. In this study, the expression of LCAL4 long non-coding RNAs was studied in 62 biopsy samples of breast cancer patients, the results showed a significant increase in LINC01416 expression in cancer samples compared to normal samples. The relationship between LncRNAs and the pathophysiology characteristics of patients was investigated, and for LINC01416, its expression was higher in tumor samples and higher in grade 1 samples. Changes in the expression of the two studied genes in relation to different subtypes in breast cancer were investigated, and no significant correlation was obtained. LCAL4 LncRNA. In the samples of the patients studied in this research, a seven-fold increase in the expression of LncRNA LCAL4 was observed compared to normal tissues, which confirms the role of this LncRNA in the pathways that cause or develop breast cancer. Also, among cancer biopsies, the expression of this LncRNA was significantly higher in grade 1 and 2 samples than in grade 3 samples, which indicates its primary role in cancer, although the increased expression in grade 3 tumors compared to normal sample. Its important role can be to help tumor development in breast cancer.

On the other hand, in this study, the expression of this Lnc RNA in the studied samples was examined in terms of the presence or absence of estrogen receptor, and the results showed no significant

difference in its expression between samples with or without estrogen receptor. This indicates that the function of the gene product is not related to estrogen or progesterone receptors. According to the mentioned cases, it can be said that the examination of LCAL4 LncRNA expression as a diagnostic biomarker also showed good results in the diagnosis of Luminal B subtype.

have been conducted to investigate the expression of LCAL4 LncRNA and the importance of its role in tumor development and metastasis, and in all of them, an increase in its expression has been observed and reported. Wu and his colleagues showed that they observed increased expression of LCAL4 LncRNA and increased metastasis and proliferation of cancer tissue in the stomach (33). The significant increase in the expression of LCAL4 LncRNA observed in the cancerous tissues of our study compared to healthy control tissues emphasizes the tumorigenicity of this LncRNA. Wang et al.'s study used LCAL4 LncRNA expression assay as a biomarker for glioma diagnosis and concluded that its increase has a direct relationship with this disease as an invasive factor, and in this sense, it is in line with our results in the field of breast cancer (34). Yan et al.'s study as a biomarker in the diagnosis of esophageal cancer is consistent with our study in the field of breast cancer (35). In the study of Liu et al it was shown that in lung cancer, a factor calle CAFs or Cancer Associated Factors surrounds this Lnc and causes the occurrence of lung cancer, the process of its increase is in line with the studies conducted in breast cancer (36). In Sun et al.'s study, it was concluded that the increase of this Lnc can play an important role as a biomarker in the identification of Non-Small Cell lung cancer, which was consistent with the increase of this Lnc in breast cancer in our studies (37). In the study of Huanjo et al., it was shown that there is a SNP in the LINC01416 gene that acts as a HOT SPOT and increases the expression of this Lnc as a result of its binding to mir-616 and activation of the PI3/AKT pathway, which results in It causes squamous cancer, which is consistent with its increase in breast cancer in our studies (38). In the study of Chen et al., it was also shown that the increase of this Lnc causes excessive activation of the beta catenin/wnt pathway and causes pancreatic cancer, which is in line with our results in breast cancer (39). The study of Lee et al showed that the increase of this Lnc as a marker in the identification of Hepatocellular Carcinomaa along with other Lncs can play a role, which is in line with our research in the field of breast cancer (40).

Conclusion

According to the mentioned cases and the findings of this study in breast cancer, it can be concluded that LCAL4 LncRNA has a very important role in the main processes of cell cycle or cell metabolism, which loss of regulation and as a result its overexpression causes development of tumors and metastases. In the field of research, for further studies, the regulatory genes of LCAL4 LncRNA can be taken into consideration; their failure to function properly causes the loss of LCAL4 LncRNA expression control. Also, the genes that are suppressed or enhanced by this LncRNA should not be ignored. What seems obvious is that LCAL4 lncRNA plays a similar role as an oncogene rather than a tumor suppressor, as its overexpression in tumors has been observed in most of the studied cases. Finally studies on LncRNA are still in the beginning and to understand the precise and critical role of LncRNA LCAL4 more studies are needed in cancers, especially breast cancer.

Conflict of Interests

Authors declare no conflict of interests.

Acknowledgments

None.

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Citation: Behyari M, Behyari M. **Expression of Intragenic LCAL4 Long Non-Coding RNAs as a Potential Diagnostic and Prognostic Marker in Female Breast Cancer.** J Family Reprod Health 2024; 18(2): 129-39.