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Non-coding RNA NAV2-AS2 and the Risk of Lobular Carcinoma in Breast Cancer Patients ***(Running title: NAV2-AS2 and the Risk of Breast Lobular Carcinoma)***

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ABSTRACT

Background & Objective: Breast cancer is the most prevalent invasive cancer and the second major cause of cancer deaths in women after lung cancer. Awareness of the symptoms and need for screening are essential to reducing the risk of breast cancer. Despite its high incidence rate, the molecular biology of breast cancer remains unknown. The present study aimed to investigate the expression of non-coding RNA (lncRNA) NAV2-AS2, which is located on chromosome 11p15.1 as a member of neuron navigator gene family, in breast cancer.

Materials and Methods: Total RNA was extracted from 40 tumors and 40 normal tissues using the RNA extraction kit. After cDNA synthesis using Takara cDNA synthesis kit, the expressional analysis of the AS2 gene was evaluated by real-time polymerase chain reaction (PCR).

Results: AS2 was overexpressed in 45% of the tumor samples compared to the normal samples. Statistical analysis showed significant correlations between AS2 gene expression, lobular carcinoma, and histology grade in situ breast cancer. In other words, the increased expression of this non-coding RNA was associated with a higher risk of lobular carcinoma and disease grade.

Conclusion: According to the results, NAV2-AS2 genes may be effective in the tumor genesis and progression of breast cancer.

Introduction

After puberty, a woman's breast consists of fat, connective tissue, and thousands of lobules, which are the minuscule glands that produce milk for breastfeeding. Very small tubes, or ducts, carry the milk toward the nipple. In cancer, the cells in the body multiply uncontrollably, and the subsequent excessive cell growth causes cancer. Breast cancer usually begins in the inner lining of milk ducts or the lobules that supply them with milk. From there, the disease could spread to the other parts of the body [1].

Breast cancer is a complicated, heterogeneous disease that is molecularly categorized into four distinct subtypes, including luminal A, luminal B, *HER2* overexpression, and triple-negative breast cancer [2]. Based on these classifications, various therapies are performed on breast cancer patients. However, the metastatic potential may vary even in the similar subtypes of cancer. To improve the prognosis of breast cancer, it is crucial to identify new, efficient therapeutic targets for effective disease management.

Long non-coding RNAs (lncRNAs) have been introduced as the deregulated factors in various cancers [3]. LncRNAs, which are longer than 200 bases and contain more than 90% of the genome, are involved in different cell mechanisms. Growing evidence has suggested the function of lncRNAs in various cancer features, such as cell proliferation, metastasis, and apoptosis. Increased knowledge of the role of lncRNAs in physiological and pathological processes has highlighted their potential use as diagnostic biomarkers, as well as targets for the novel therapeutic approaches toward malignancies [4]. Although some studies have defined different lncRNAs as tumor suppressors or oncogenes in tumorigenesis, it is of utmost importance to investigate the role of other lncRNAs in different cancers.

Neuron navigator 2 (*NAV2*) is one of the three members of the neuron navigator family, which is involved in neurogenesis and tumorigenesis [5]. NAV2 protein contains various functional domains, including the calponin homology (CH) domain, four coiled-coil (CC) domains, a cytoskeletal interacting domain (CSID), and an AAA domain [6]. The functional domains are involved in various cellular processes, including protein degradation, signal transduction, regulation of gene expression, membrane fusion, microtubule dynamics, and cell migration. Previous findings have confirmed that *NAV2* affects cell migration through adjusting microtubule dynamics [7, 8], leading to basement membrane breakdown, formation of actin stress fiber, and metal matrix protease secretion. Furthermore, *NAV2* regulates the cytoskeleton, which affects cell-cell and cell-matrix adhesion, facilitating tumor cell invasion and metastasis [8, 9]. Previous studies have denoted that *NAV2* plays a key role in cancer and metastasis through cofilin, which is an essential regulator of actin dynamics and the targeting factors in APC/Wnt/β-catenin signaling pathway. On the other hand, few studies have assessed

NAV2 Antisense RNA2 (*NAV2-AS2*), which is located in the minus strand of the *NAV2* gene.The present study aimed to investigate the mRNA expression level of *NAV2-AS2* lncRNA in breast cancer and elucidate their correlation with the indices of poor prognosis in breast cancer patients.

Materials and Methods

In this study, tumor and paired normal tissues were obtained from 40 breast cancer patients by Iran National Tumor Bank, which has been founded by Cancer Institute of Tehran University of Medical Sciences for cancer research. The demographic characteristics were based on the seventh edition of Union International Cancer TNM classification guidelines. The study was performed after obtaining informed consent from all the recruited patients.

RNA Extraction, cDNA Synthesis, and qRT-PCR

Using TriPure RNA extraction reagent (Roche, Nutley, NJ), RNA was extracted from all the tissues. The quality of RNA was assessed by gel electrophoresis and nanodrop. PrimeScript First Strand cDNA Synthesis Kit (Takara, Japan) was used for cDNA synthesis. To assess the mRNA expression level, specific primers were designed using Primer3 online software, and cDNA was amplified by qRT-PCR using the SYBR green method (SYBR green mix, YTA, Iran) on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Philadelphia, PA, USA).

Correlation of the Overexpression of AS2 with Histology Grade and Lobular

The following optimal thermal conditions were applied: 10 minutes at the temperature of 95°C, 45 cycles of 15 seconds at the temperature of 95°C, 30 seconds at the temperature of 60°C, and 45 seconds at the temperature of 72°C. Data of real-time polymerase chain reaction (PCR) were normalized using glyceraldehyde 3-phosphate dehydrogenase (Table 1) [Liu L-L et al., 2015], and the ΔΔCT method was applied for the fold change evaluation of the gene expression in the tumor samples and comparison with the normal tissues. All the reactions were performed in triplicate.

Statistical Analysis

Data analysis was performed in SPSS version 23 (SPSS, Chicago, IL) using $\chi 2$, Fisher's exact test, independent sample t-test, and analysis of variance (ANOVA) to determine the correlations of mRNA expression level with the clinical and pathological data. P-value of less than 0.05 was considered statistically significant.

Results

Study Population and Clinical Demographic Data

In total, 40 female patients with breast cancer, who had received no treatment, were enrolled in the study. Mean age of the patients was 49.55±13.03 years. The size of tumor samples was 1.5-10 centimeters. Clinical and pathological features of the patients are presented in Table 2.

Carcinoma in Situ Histology in Breast Cancer

The relative and comparative real-time PCR on *AS2* expression showed the overexpression of this lncRNA in breast cancer. Mean *AS2* gene expression was 1.1±0.9 in the breast cancer tissues, and the gene was significantly overexpressed in approximately 45% of the tumor samples (18 out of 40 samples). Increased level of *AS2* mRNA expression was significantly correlated with various indices of poor prognosis, including lobular carcinoma in situ histology (P=0.014) and histology grade (P=0.023).

Discussion and Conclusion

Breast cancer is a highly heterogeneous disease, and selecting the appropriate treatment to improve its prognosis is rather complex [10]. The molecular investigations regarding the initiation, progression, and metastasis of cancer have successfully elucidated the mechanism of this disease, as well as the most effective target therapies [11]. LncRNAs have been assessed extensively after demonstrating the different regulating roles of non-coding RNA HOTAIR in cancer. The correlation between lncRNA and various indices of prognosis could definitely lead to finding the main regulators of the master genes in cancer and targeted therapies. In the present study, the expression level of *NAV2-AS2* was evaluated to verify their possible role in breast cancer progression. According to the findings, the overexpression of *AS2* was significantly associated with lobular carcinoma in situ histology and histology grade.

NAV2 is located on chromosome 11p15.1 and is identified with other aliases, such as *UNC53H2*, *POMFIL2*, *HELAD1*, and *RAINB1* [12]. This gene was initially considered to be an all-trans retinoic acid-responsive gene in neuritis outgrowth [6]. Recently, the overexpression of *NAV2* has been highlighted in 16 out of 20 colon cancers [13]. Examination of the molecular mechanism of *NAV2* overexpression has indicated its role in the migration and invasion of colon cancer through F-actin polymerization and the SSH1L/cofilin-1 pathway [14]. Moreover, several studies have confirmed the role of cytoskeleton in epithelial-mesenchymal transition and cancer [15-17]. In this cohort study, we analyzed the *NAV2-AS2* lncRNA, and the overexpression of *NAV2-AS2* was observed in the breast cancer patients.

Previous studies have reported variable lncRNAs overexpression in breast cancer. For instance, Deng J et al. (2017) claimed that the overexpression of HOTAIR (a well-known lncRNA) is a key regulator of the main mechanisms of cancer stem cells, including proliferation, colony formation, invasion, and self-renewal capacity, in breast cancer [18]. Furthermore, the overexpression of RNA-H19 has been shown to promote breast cancer cell clonogenicity and migration [19]. In another study, Huang NS et al. (2016) stated that the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) as the lncRNA was up-regulated in breast cancer [20]. In line with these studies, our findings indicated that *NAV2-AS2* was overexpressed in breast cancer in correlation with the histology grade of the disease. In our cohort, 64% of the patients had grade II breast cancer with the overexpression of *NAV2-AS2*.

Intriguingly, the overexpression of this lncRNA was statistically correlated with lobular carcinoma in situ (LCIS) histology; as a result, we predicted that *NAV2-AS2* could be a novel lncRNA to be used as a diagnostic biomarker for breast cancer since LCIS could indicate the increased risk of cancer in the future. Such extrapolations require further *in-vitro* and *in-vivo* investigations in order to determine the exact mechanisms of how the deregulation of antisense RNA could alter the expression of *NAV2* and the possible tumorigenic role of this perturbation.

In conclusion, our study demonstrated a novel lncRNA in breast cancer, the overexpression of which has a significant association with various indices and carcinogenesis of this cancer. In addition, this lncRNA could be a novel biomarker to diagnosis breast cancer in the early stages considering its significant correlation with LCIS.

Conflicts of interest: None declared.

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Table 1. Primer Sequences Used for qRT-PCR

Gene	Forward Primer	Reverse Primer
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATATCCACT
AS2	ATTCTTCTTGCCCGACGTG	ATTCCCTGTGGTCTGGTGATG

Table 2. Clinical and Pathological Data of Patients

		N	*NAV2-AS2* Expression
Age (year)	>50	20	.402
	<50	20	
Tumor Size	0-2	2	0.677
	2-5	28	
	5<	8	
Pathological (T)	Tx	2	0.34
	T1a	2	
	T2	25	
	T3	9	
	T4	2	
Pathological (N)	Nx	4	0.20
	NOITC	2	
	NO(NOC)	12	
	N1	13	
	N2	7	
	N3	2	
Clinical Metastasis	Mx	3	0.378
	M0	35	
	M1	1	
	M Pending	1	
Histology Grade	I	7	0.023*
	II	18	
	III	8	
	X	7	
Lobular Carcinoma in Situ Histology			0.014*

*The clinical factors (age, tumor size, tumor type, tumor classification) show the tumor invasion of the surrounding tissues, classification of the lymph nodes that demonstrate the spread of cancer to the surrounding tissues, classification of metastasis, probability of the dissemination of metastases to other areas, and histology grade and lobular carcinoma in terms of number and their association with NAV2-AS2 expression.