The Role of Natural of AntisenseTranscription *HAGLR,LCMT1AS, NAV2AS5,TSIX* in Breast Cancer

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Abstract

Backgraound: lncRNAs (longnon-codingRNAs), has avarious important molecular and cellular functions and natural antisense transcripts (NATs), complementary to protein-coding or non-coding. RNA sequences are important regulators of gene expression drew a great attention recent years to uncover their importance for diagnostic, prognostic and therapeutic purposes; and their dysfunctions leads to diseases including cancer.

Aims: The aim of this study was to investigate important roles of lncNATs *TSIX, HAGLR, LMCT1AS* and *NAV2AS5* genes in several tumorigenic processes in breast cancer.

Materials andMethods: In this study we used ATCC normal cell lines (*CRL4010,CRL8798*) and cancer cell lines (*MCF7,MDA-MB231,CRL2329*) that subjected. To examine through RNA isolation,cDNAconversion, semiquantitative (by agarose gel and ImageJ program) and quantitative RT-PCR for gene expression analyses.

Results: Our results have been shown that *TSIX, HAGLR, LMCT1AS* and *NAV2AS5* genes have differential expression pattern in both normal and breast cancer cell lines.

Conclution: from the data of our results we concluded that these genes have important role in biological procesess of breast cancer in addition their impotnace in treatment and therapeutic purpus.

1- Introduction

Non-coding RNAs (ncRNAs) have gained another dimension with new findings. Long-coding RNAs (lncRNAs) are a broad and diverse class of transcriptional RNA molecules that do not encode proteins with more than 200 nucleotides in length. Many lncRNAs, including transposons, pseudogenes and simple repetitions that are biologically important functional regulators, are transcribed from genomic regions called 'trash' [1]. LncRNAs are becoming more and more important as a research topic related to cancer. So far, many lncRNA molecules have been identified that have roles in cancers. Some LNCRs act as tumor suppressors, while others behave like oncogenes. It is reported that many lncRNAs such as *MALAT1, H19* and *HOTAIR,* which act as oncogenes in many cancers such as lung cancer, colon cancer, liver cancer, breast cancer etc. [2]. There are many lncRNAswhich act as tumor suppressors. A study [3]reported that MEG3 acts as a tumor suppressor in many types of cancer. lncRNAs act by enhancing or decreasing expression levels by binding to noncoding or protein-encoding RNAs. Natural antisense transcripts (NATs), complementary to protein-

coding or non-coding RNA sequences are important regulators of eukaryotic gene expression. Natural antisense transcripts (NATs) that bind to long non-coding RNAs (lncRNAs) called lncNATs.There was not enough studies yet for a new class of lncNATs. *TSIX*acted as a new regulator expression which stabilizes the mRNA[7],*XIST*and *TSIX*transcription regulated by X to Autosome ratio thatstabilize the transcription factor [4]. Dysregulation of lncRNAsin various cancer cells serve as oncogenes or tumor suppressors. Long non-coding RNA HOXD-AS1 have role in the development of different cancers including neuroblastoma and breast cancer. *HOXD-AS1*regulates proliferation in Prostate Cancer [5].We hypothesized that, *TSIX*, *HOXD-AS1/HAGLR*, *LCMT1AS* and *NAV2AS5*antisense might be regulated by each other for several biological processes in cancer. The other hypothesis for selecting these four type of genes in our study is that all of them may have roles as tumor suppresser. We checked *TSIX*, *HOXD-AS1/HAGLR*, *LMCT1AS* and *NAV2AS5* antisense transcripts in normal cells (CRL4010, CRL8798) and cancer cells (*MCF7*, *MDA-MB-231*, *CRL2329*). These are commercially available cell lines from the ATCCUntil now, it is still a matter of debate whether *TSIX*is acting as an oncogen or as a tumor suppressor. In many biological processes, there are many lncRNAs targeting proteins that bind to DNA [6]. LncRNAs act with proteins that bind to DNA to regulate the transcription of DNA epigenetically. The interaction of TSIX with long coding RNAs is still open to research.Therefore in this thesis, we aimto find the interactive role between lncRNA *TSIX*, *HOXD-AS1/HAGLR*, *NOV2AS5* and *LCM1AS* in cancer. Also we would like to address the respective role of *TSIX*, *HOXD-AS1/HAGLR*, *NOV2AS5* and *LCM1AS* in cancer.This study will open a new avenue in cancer therapy by understanding their oncogenic or tumor suppressive role and also will help to find new therapeutic targets. Long non-coding RNAs (lncRNAs) have a major sequenceeffect on healthy problem in all countries and predominantly affects different .Long non-coding RNAs (lncRNAs) it is the most common type of gene regulation in women and man responsible from the most of the cancer-related to gene expression and regulate sequence of noncoding RNA.A lot of work has been done about the types and biology of cancers for many years. Studies have been showed that the cancer cell loses their death mechanisms and behaves like pseudo embryonic cells. lncRNAsare thought to nearly 30,000 due to in humans transcript, the non-coding transcriptome the major part of lncRNA ,invented of lncRNA transcripts account was still done as preliminary stage.The human genome consists of more than 2 m of linear DNA that is inserted into a three-dimensional structure in the nucleus of each cell. Molecular biology of the central doctrine suggested that the flow of genetic information is from DNA to RNA and from RNA to protein. However, in the last decade this dogma has gained new dimensions with the discovery of unencoded RNAs (ncRNAs) [7].

Cancer

Breast cancer considered one of the common cancers. In spite of developing treatment methods, breast cancer cannot be treated effectively. Therefore, the identification of novel biomarkers that will be important in the treatment and diagnosis of breast cancer was great interest. Cancer was a leading cause of death through out of the world ,from a total of 58 million death world wide in 2005, cancer accounts for 7.6 million or 13% of all death.

Cancer defined as a genetic term which encompasses more than 100 disease that affected any part of the body. The world cancer only applied to malignant tumours, there fore by definition, all cancer were malignant tumours. Tumours may be either benign or malignant and believed to emerge only when immune surveillance fails. The two important difference between benign and malignant tumours invasion and spread [8]. Breast cancer was a major health problem in all countries and predominantly affects women population. In United States, breast cancer was estimated that 300-400 new cases related deathwas occur. Also according to 2014 cancer statistics in Turkey, breast cancer (24.9%) was the most common cancer of women in all age groups [9].

2- Materials and Methods

Collection of cell lines:

In this study, 1 ml of cell line samples were collected in order to isolatate RNAs from 7different cell lines. Samples were stored at -80°C in ependorf tubes to be used for RNAisolation.

Table 1: Information of 7 cell line patients

No.	NAME OF CELL	Name of tissue	Biosafety Level	Organism	Cell Type	nationality
1	*CRL2329*	Breast cancer	1	*Homo sapiens*	Lobular	U.S
2	*CRL4010*	Breast normal	1	*Homo sapiens*	epithelial	U.S
3	*MDAMB*	breast cancer	1	*Homo sapiens*	Ductal	U.S
5	*CRL8798*	Normal breast	1	*Homo sapiens*	Lobular	U.S
6	*MCF7*	Breast cancer	1	*Homo sapiens*	Tumor	U.S

1: stage of tumor cancer cell Homo: type cell line*U.S: Unite State

Cell Culture

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum , 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified atmosphere containing 5% CO2 at 37 °C, at Medical Biology Department Cell Culture Lab., Gaziantep University.

Primer Design

For this study, appropriate synthetic primers were designed for intron regions of *Tsix* and *HAGLR and LCMT1AS and NAV2AS5* genes by using NCBI/Primer Blast database. Primer sequences, length, annealing temperature, GC content and PCR product length of primers designed for intron sequences of *HAGLR and LCMT1AS and NAV2AS5* genes were shown in Table (3) in a detailed manner.

Table 3: Primer sequences, length, annealing temperature, GC content and PCR product length of primers designed for intron sequences of *HAGLR and LCMT1AS and NAV2AS5* genes.

Genes	sequence (5'->3')	Primer lenght	temperture (°C)	GC%	PCR product(bp)
TSIX	GTTGCATCAGCTGTCCTCCT	17	57.75	64.71	221
	AAAAAGGGGTTGGGGTAGG	20	61.90	60.00	
HAGLR	ACCAGACCTACTCTTCCGCT	20	59.88	55.00	246
	GGGAAGAGCCAAGTCAGAC	20	60.03	55.00	
LCMT1AS	ATCTGGTGAGCCAGGTAGGA	20	59.53	55.00	207
	GGGAAGAGCCAAGTCAGAC	21	59.44	52.38	
NOV2AS	CCCACTGTGAGAACCCCTTC	20	59.96	60.00	209
	GAGACCCATGCCAGTGTGTG	20	60.96	60.00	

RNA Isolation from cell line :

After cells reach 80-90% density, they were removed for RNA isolation under appropriate conditions. RNA was extracted from cell culture using RNA isolation kit (Roche, Mannheim, Germany) kit.RNA isolation protocol have been done as follows;The cells with appropriate density were removed with Trypsin, and DMEM containing FCS added to stop the effect of trypsin. Cells centrifuged at 3500 rpm for 5 min. The supernatant removed without touching the pellet. The remaining pellet resuspended in 200 µl PBS.- Add 400 µl of Lysis Buffer to this mixture and vortex for 15 seconds. The whole mixture transferred to filter tubes, centrifuged for 30 seconds at 9200 rpm. The lower part discarded.Add 100 µl (10 µl DNAse and 90 µl DNAse incubation buffer) to the filtered tubes and wait at room temperature for 45 minutes. Add 500 µl Wash Buffer I and centrifuged at 9200 rpm for 30 seconds. The lower part discarded.Add 500 µl Wash Buffer II and centrifuged at 9200 rpm for 30 seconds. The bottom tube replaced with the new one.Add 200 µl of Wash Buffer II and centrifuged for 2 minutes at 11800 rpm. The lower tube discarded and a new tube wasinserted.Add 50 µl of Elution Buffer and wait for 1 minute at room temperature. Centrifuge at 9200 rpm for 1 minute. The filtered tube discarded. Measurements were made on the NanoDrop 1000 to determine the amount of RNA.RNAs were stored at -80 ºC until the working period.

3- Statistical Analysis

For statistical analysis Graph Pad Prism 6 was used. Wilcoxon Signed Rank Test were performed for data analysing and P values ≤ 0.05 was considered as statistically significant.

4- Results

Gene expression results of *TSIX ,HAGLR,NOV2AS5,LCMT1AS* in different cell linesThe expression level of *TSIX ,HAGLR,NOV2AS5,LCMT1AS* has been analyzed in 7 different human cell line. Gene expression analyzes were performed by Real-Time PCR methods (Figure 1). *TSIX* founded to be show expression in breast cancer cell line.

	GEN	İntDen	GEN/GAPDH
1	GAPDH	53877	
2	TSIX	55204	1,02463018
3	HAGLR	53476	0,99255712
4	NAV2AS5	55795	1,03559961
5	LCMT1AS	49157	0,91239304

Figure1: by Imag j program can detect concentration level of genes band and compare with *GAPDH*housekeeping gene Expression levels of *TSIX ,HAGLR,NOV2AS5,LCMT1AS* and *GAPDH* genes by RT-PCR.in breast cancer cell line.

LCMT1A LCMT1AS HAGLR TSIX GAPDH

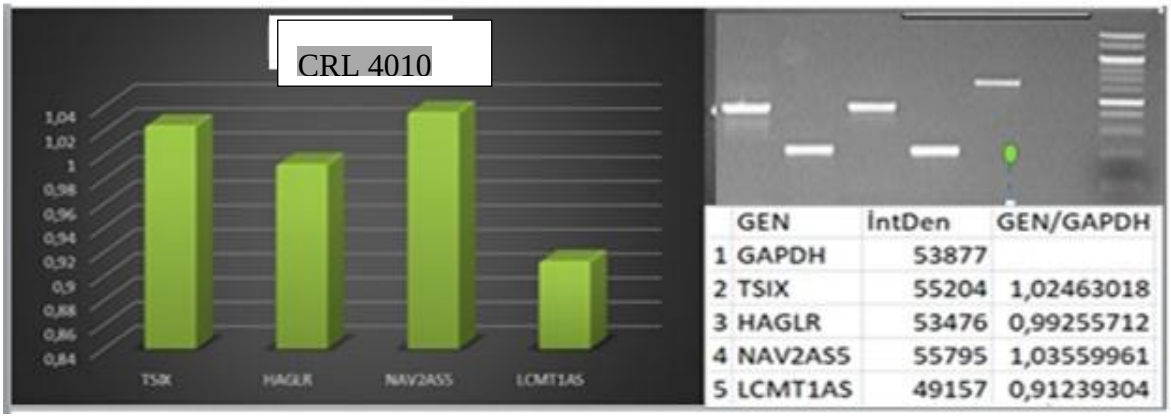

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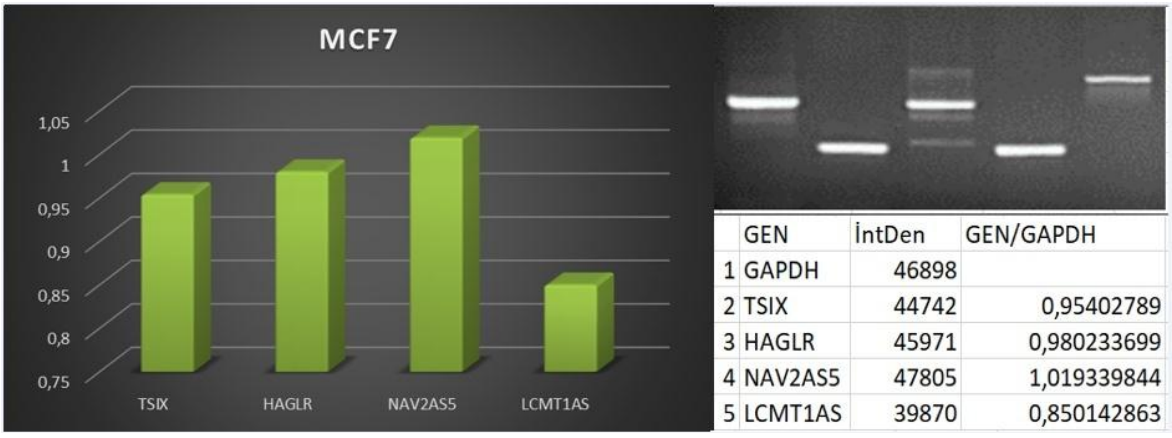
Figure 2: Expression levels of *TSIX ,HAGLR,NOV2AS5,LCMT1ASand GAPDH* genes by Real Time-polymerase chain Reaction (RT-PCR) in breast cancer cell line CRL4010 normal cell line.

LCMT1A LCMT1ASHAGLRTSIX GAPDH



	GEN	İntDen	GEN/GAPDH
1	GAPDH	46898	
2	TSIX	44742	0,95402789
3	HAGLR	45971	0,980233699
4	NAV2AS5	47805	1,019339844
5	LCMT1AS	39870	0,850142863

Figure 3: Expression levels of *TSIX ,HAGLR,NOV2AS5,LCMT1AS* and *GAPDH* genes by Real Time-Polymerase Chain Reaction (RT-PCR) in normal cell line Htert-HME1.The concentration band of *NOV2AS5* higher than the other one its pure band in the normal cell line .

LCMT1A LCMT1ASHAGLR TSIX GAPDH

	GEN	İntDen	GEN/GAPDH
1	GAPDH	55563	
2	TSIX	53680	0,966110541
3	HAGLR	38866	0,699494268
4	NAV2AS5	52048	0,936738477
5	LCMT1AS	39863	0,717437863

Figure 4: Expression levels of *TSIX* ,*HAGLR,NOV2AS5,LCMT1AS* and *GAPDH* genes by Real Time-Polymerase Chain Reaction (RT-PCR) in breast Cancer cell line,*MDA-MB*-231.The concentration of *NOV2AS5* higher than the other genes.

Expression level of HAGLR in breast cancer cell lines

Expression level of *HAGL*Rhave been shown in 5 different cell lines. Gene expression analyzes were performed by Real-Time PCR methods (Figure 6). *HAGLR* has been shown to have the highest expression level in *CRL2329* cells for breast cancer followed by expression level in the *MCF7,MDA-MB-231,CRL2329,CRL4010* cell line.

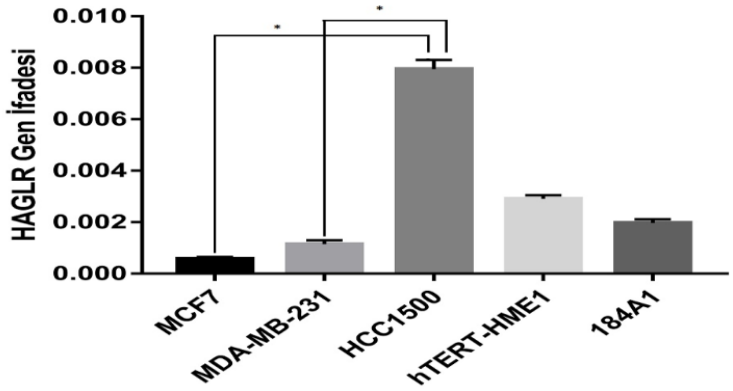


Figure 6: *HAGLR* has been shown to be expressed mostly in breast cancer and control group was found to show expression cancer and normal cell line,the level of gene expression of cancer cell line lower than the normal cell line.

Expression level of TSIX in breast cancer cell lines

Expression level of TSIX has been shown in 5 different cell lines. Gene expression analyzes were performed by Real-Time PCR methods (Figure 7).

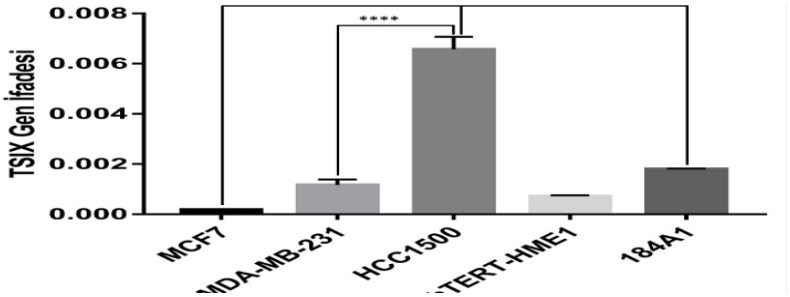


Figure 7: *TSIX* has been shown to be expressed mostly in breast cancer and control group was found to show expression cancer and normal cell line.*CRL2329* is the *HCC1500* traditionaly name of cell line cancer cell line can higher than the normal group.

Expression level in *NOV2AS5* of breast cancer cell line

Expression level of *NOV2AS5*has been shown in 5 different cell lines. Gene expression analyzes were performed by Real-Time PCR methods (Figure 8). *NOV2AS5*has been shown to have the highest expression level in *CRL2329* cells for Breat cancer followed by expression in the *MCF7,MDA-MB-231,CRL2329,CRL4010* cell line. *NOV2AS5*was found to be the lowest in *MCF7,MDA-MB-231,CRL2329,CRL4010*, control group and cancer group.

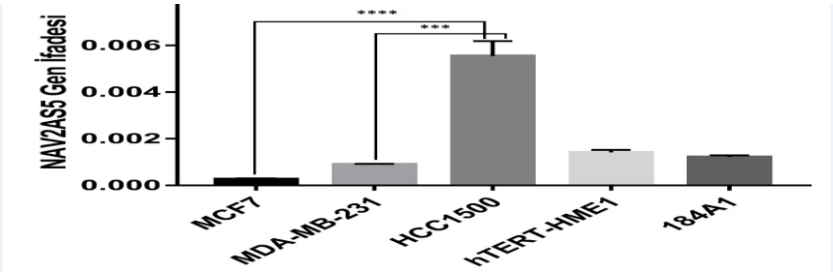


Figure 8: *NOV2AS5* has been shown to be expressed mostly in breast cancer and control group was found to show expression cancer and normal cell line.

The expression level of *LCMT1AS* in breast cancer cell lines

Expression level of *LCMT1A*Shas been shown in 5 different cell lines. Gene expression analyzes were performed by Real-Time PCR methods (Figure 9).*LCMT1AS*has been shown to have the highest expression level in CRL2329 cells for breast cancer following expression in the *MCF7,MDA-MB-231,CRL2329,CRL4010* cell line. *LCMT1AS*was found to be the lowest in *MCF7,MDA-MB-231,CRL2329,CRL4010*, control group and cancer group.

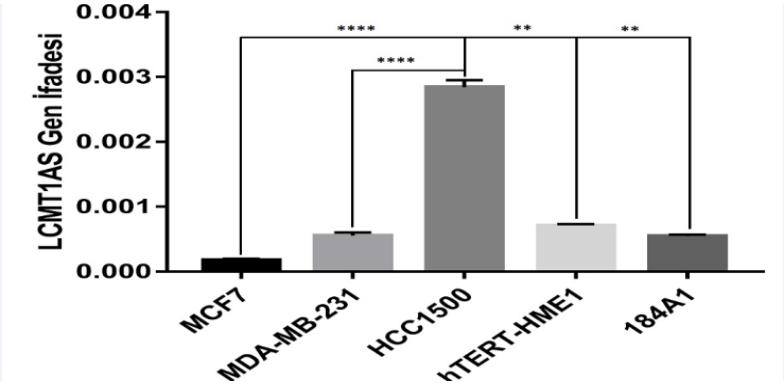


Figure 9:*LCMT1AS5* has been shown to be expressed mostly in breast cancer and control group was found to show expression cancer and normal cell line.

5- Discussion:

LncRNAs (long non-coding RNAs), with various important molecular and cellular functions and natural antisense transcripts (NATs), complementary to protein-coding or non-coding RNA sequences are important regulators of eukaryotic gene expression drew a great attention recent years to uncover their importance for diagnostic, prognostic and therapeutic purposes because of their dysfunctions leads to diseases including cancer. Earlier NATs were described as lncRNAs. This convergence between NAT and lncRNA determination raised confusion and gradually started to be disappeared withthe increasing knowledge. Specific pcGen (protein coding Gene) regulation by their corresponding ncNATs (non-coding NATs) has been reported. *TSIX* was a new regulator of collagen expression which stabilizes the collagen mRNA. *HOXD-AS1/ HAGLR*was a critical regulator for carcinogenesis and metastasis in different types of cancers. *LCMT-1*was a negative regulator of Akt proto-oncogene. Some information was available related with *NOV2AS1* gene. This four type of genes regulate cancer cell. We performed comparison with control group and cancer groups. The tumor supprusor genes(*TSIX, HAGLR, LCMT-1* and *NOV2AS1*)were regulated downwards in cancer cell line.

The aim of our study was to investigated the level of mRNA expression and screening of gene by Jel electrophoresis of *TSIX, HAGLR, LCMT-1* and *NOV2AS1* genes in breast cancer and normal group.

Studing the molecular mechanisms of cancer cells will be a base research for other related investigations and will create opportunities for new cancer therapy specially breast cancer. The strategies regarding investigating these genes related to cell cycle will be beneficial for understanding anti-tumoral effect for target organs.*TSIX* was a tumor

suppressor gene . It was identified by the analysis of accumulated transcripts including non coding histone methyltransferase, which is responsible for trimethylation of the lysine of histone H3 and may have a role in the regulation of mRNA transcriptionfactor (suppressing tumor development) [10]. Many studies suggested the potential of gene expression models to distinguish between histologic subtypes effect to expression level from cancer group and normal group. Gene expression level of tumor suppressor genes were identified by Real–Time PCR analysis.The seven type of cell lines of breast cancer and lung cancer were statistically comparable to the control group of their normal cell lines.It has been understood that under normal condition there was over expression from cancer cell lines incomparison to the normal group. The expression level of *TSIX, HAGLR, NOV2AS5, LCMT1AS* and *GAPDH* genes in normal breast and cancer cell lines have been demonstrated by RT-PCR analysis. *NOV2AS5* has higher expression than other genes.In our study, the expression level of *TSIX,HAGLR,NOV2AS5,LCMT1AS* genes was decreased (downregulated) as shown in Figures 6, 7 ,8,9 and statistically were significant ($p > 0.05$). This results agree with results [11] that reported the downregulated expression of *TSIX, HAGLR, NOV2AS5, LCMT1AS.* Functional inactivation of *TSIX* in lncNAT mayfacilitated the development of an regulator tumor behavior [12]. The role of *TSIX* and *HAGLR* in chromatin modification, the gene expression pathways disrupted by the activation of antisence that may lead to new treatment strategies for different tisseus [13]. The balance between histone acetylation and deacetylation serves as a key epigenetic mechanism for gene expression, DNA repair, developmentalprocessesandtumorigenesis[14]. Thus, any reason to make this imbalance can lead to abnormal cell function, even tumor genesis [15]. Another sutudy[16] reported that MOF an acetyltransferase of H4K16 might be involved in the pathogenesis of renal cell carcinoma, and this epigenetic change might be a new CA9-independent RCC diagnostic marker. Amicro RNA expression study suggested that the involvement in tumor development and tumor progression including metastasis [17]. Analysed distant metastases with primary tumours and founded a distinct miRNA signature at metastases. Some of the primary tumour samples clustered together with the distant metastasis, so that these primary tumourshave a metastasis-specific signature[18]. In this study, mutated regions of *TSIX, HAGLR, NOV2AS5* and *LCMT1AS* genes were analyzed by q PCR roter gene and sequence analysis. However, expression was observed in these regions. *TSIX* gene expression was compared from two group desease and a normal group.

We analyzed by ImageJ Programe is comprised of two types of bromodomains in this study such as domain 1; and random region of *TSIX, HAGLR, NOV2AS5* and *LCMT1AS* genes.

6- Conclusion

In conclusion, the expression level of *TSIX, HAGLR, NOV2AS5,LCMT1AS* genes had differential significant downregulated. Studing the molecular mechanisms of a tumor suppressor of gene had aided the development of molecular-targeted therapy for breast cancer. In order to study the molecular mechanisms we investigated between breast cancerand normal breast. Further analysis is needed.We have prepared the ground work for our next study ,tumor suppressure effect or not , we found a result of this, for next study we can check the cell cycle for growth or not , or we can give siRNA for inhibit gene expression or give spesific miRNA for block gene target.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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دور النسخ الطبيعيه غير الحسيه للجينات HAGLR، LCMT1AS، NAV2AS5، TSIX في سرطان الثدي

الخلاصه

IncRNAs (الرناالطويلة غير المشفرة) لها وظائف الجزيئية والخلوية الهامة والنسخ الطبيعية المضادة (NATs) هي مكملة للبروتينات المشفرة او غير المشفرة. والقاعده الناتيروجينيه لـــ RNA لها اهميه في اظهار الجينات.، والتي لقيت اهتماما كبيرا في السنوات الأخيرة لأهميتها التشخيصية والعلاجية ،وان أي خلل في وظيفيتها يؤدي إلى أمراض بما في ذلك السرطان.

الهدف: الغرض من البحث هي اكتشاف دور واهميّة االجيناتlncNATsTSIX, HAGLR, LMCT1AS NAV2AS5 في عملية تكوين الأورام في سرطان الثدي.

الموادوالطرق المستعمله: المواد والطرق المستعمله في بحثنا هذا هي خلايا الطبيعيه (ATCC(CRL4010, CRL8798 وخلايا مسرطنه ,MCF7 (MDA-MB-231, CRL2329)، وتم استخلاص كل منRNA و cDNA، ولأظهار الجينات تم االتحليل بواسطه استخدام برنامج (agarose gel andImageJ program) semiquantitative والـــ quantitative RT-PCR.

النتائج: أظهرت النتائج ان الجينات TSIX, HAGLR, LMCT1AS، NAV2AS5 لها أنماط مختلفه الظهور في كل من الخلايا الطبيعيه والخلايا المسرطنه لثدي.

الاستنتاج: استنتجنا من هذه الدراسه او البحث ان لهذه الجينات لها اهميه بالغه في العمليات البايولوجيه للسرطان الثدي اضافه الى أهميتها لأغراض العلاجيه.

الكلمات الداله: خلايا الطبيعيه ،خلايا الثدي المسرطنهRT-PCR ، agarose gel andImageJ program.