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Overexpression of long non-coding RNA MCM3AP-AS1 in breast cancer tissues compared to adjacent non-tumour tissues

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ABSTRACT

Background: Altered expression of several long non-coding RNAs (lncRNAs) has been described in numerous malignancies, including breast cancer, and some may have a role in carcinogenesis. We hypothesised differences in the expression of lncRNA MCM3AP-AS1 in breast cancer tissues compared to nearby healthy tissues and potential links with clinical features.

Methods: We tested our hypothesis in 102 pairs of breast cancer tumours and adjacent non-tumour tissues from female patients. After RNA extraction, cDNA synthesis was performed for all specimens. The differential gene expression was assessed using Quantitative Real-Time PCR Technique.

Results: There was a significant overexpression of the lncRNAs in tumour tissues as compared with their adjacent non-tumour tissues ($P < 0.001$). Expression was significantly linked with the tumour oestrogen receptor expression ($P = 0.023$) and tumour progesterone receptor expression ($P < 0.001$). ROC analysis showed an AUC of 0.67 (95% CI 0.60–0.75) ($P < 0.001$) with sensitivity and specificity of 58% and 76%, respectively

Conclusion: The lncRNA MCM3AP-AS1 may be a novel breast cancer lncRNA with high expression levels in breast cancer patients' tissue. Further investigations are needed to confirm its uses as a potential molecular marker and therapeutic target.

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Introduction

Although breast cancer remains a significant threat to human health due to its high mortality rates, progress in its recognition at early stages and evolution of breast cancer therapies have led to an improvement in the relative survival rate and outcomes [1–3]. However, the heterogeneity of breast cancer and the involvement of diverse risk factors in the process of tumour formation and treatment resistance have challenged the methods of recognition and therapy of breast cancer patients. Gene expression patterns can determine three major breast cancer subtypes, based on the expression of oestrogen and progesterone receptors (OR+, PR+), the human epidermal growth factor receptor 2 (HER2+) and triple-negative breast cancer, the absence of ORs, PRs and HER2 expression (i.e. OR-, PR-, HER2-) [4–6].

Long non-coding RNAs (LncRNAs) are a group of RNAs of length ranging from 200 bp to 100 kb that fail to encode proteins [7,8]. The classification of lncRNAs based on their position on the genome has led to the identification of a group accounting for 32% of human genomic lncRNAs, i.e. antisense lncRNAs. LncRNAs participate in pathways that lead to the initiation, progression, metastatic functions, and resistance to malignancy treatments through various mechanisms. Currently, oncogenic and tumour suppressor lncRNAs are likely to be involved in the initiation or prevention of breast cancer. Moreover, studies on breast cancer suggest that some of the lncRNAs can be involved in the regulation of gene expression at different transcriptional, post-transcriptional, and epigenetic levels through participating in interactions between RNA-DNA, RNA-RNA or proteins and interfering with the transcription process [9–15].

One of the essential elements of pre-replication complexes is minichromosome maintenance (MCM) proteins that are composed of six-component complexes (MCM2-MCM7) by DNA helicase activities. These complexes are involved in the initiation and extension processes of DNA replication and lead to restrictions of DNA replication to once per cell cycle. Minichromosome maintenance complex component 3-associated protein (*MCM3AP*) is located at 21q22.3, and codes for a protein that binds to and acetylates MCM3 through acetyltransferase functions. The interaction of MCM3AP with MCM3 is crucial for not only its entry into the nucleus but also its binding to chromatin. Increased *MCM3AP* expression level plays a role in inhibiting the S phase of the cell cycle, and its

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regulatory role as a tumour suppressor gene with a low level of expression has been proved in breast cancer. MCM3AP-AS1 is a long non-coding RNA antisense1 for *MCM3AP* [16–19]. The roles and regulatory mechanisms of lncRNA MCM3AP-AS1 in breast cancer are unclear.

We hypothesised that the expression of MCM3AP-AS1 in tumour and adjacent non-tumour tissues of female breast cancer patients is different and is linked to the expression of ORs, PRs, HER2s.

Materials and methods

We tested our hypothesis in 102 pairs of breast tumour and adjacent non-tumour tissues collected from female patients who underwent biopsy or mastectomy surgery at Noor-Nejat Hospital (Tabriz, Iran) from October 2017 to December 2017. Excised tissues were divided: some was collected into RNase-free microtubes and immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction, the remainder for standard histopathology processing. The latter used routine H&E to define ductal or lobular carcinoma, and immunocytochemistry to define the expression of ORs, PRs and HER2s (positive/negative). The Ethics Committee of the University of Tabriz approved the study and written informed consent was obtained from each patient.

The primer pairs for each gene were designed under optimal primer conditions by Gene Runner software (version 6.5.52). After selecting the appropriate primers, their sequences were taken to BLAST at the NCBI website to control the primer specificity. Primers (Sinaclon, Tehran, Iran) were MCM3AP-AS1 forward: 5′-GCTCCTCTTCC AGTTACGGT-3′, reverse: 5′- GCGAGGGGAATTTTCTGAG TCT-3′, and β-actin forward: 5′-AGAGCTACGAGCTG CCTGAC-3′, reverse: 5′-AGCACTGTGTTGGCGTACAG-3′. TRIzol reagent (Invitrogen, Massachusetts, USA) was utilized to isolate total RNAs from breast cancer tumours and adjacent non-tumour tissues according to the manufacturer's instructions. Quantitative and qualitative evaluations of extracted RNAs were performed using NanoDrop Spectrophotometer at the A260/A280 ratio and 2% Agarose Gel Electrophoresis, respectively. The extracted RNA solution was treated with DNase I enzyme (GeneAll, Seoul, Korea) to eliminate possible DNA contamination. Regarding RNA concentration obtained after extraction, the amount of RNA in the reaction was expressed in µL. Each reaction contained RNA, 0.5 DNase I enzyme (1 U), and 0.5 µL DNase I buffer (10×). Finally, water treated with DEPC was added, as a result of which the total volume reached to 5 µL. The mixture was incubated in a thermocycler at 37 °C for 30 min. Then, 1 µL of EDTA was added to inactivate DNase I and to prevent RNA degradation at high temperatures. The obtained mixture was incubated in a thermocycler at 65 °C for 10 min.

Complementary DNA (cDNA) synthesis was performed using the Takara Synthesis kit (TaKaRa, Kusatsu, Japan). According to the manufacturer's instructions, each sample of RNA treated with DNase I enzyme was added to 3.5 µL of Mastermix Kit Taccara and 1.5 µL of DEPC-treated water. The obtained mixture was then incubated in a thermocycler at 37 °C and 85 °C for 60 minutes and 5 seconds, respectively.

Measurement of gene expression was carried out by Quantitative Real-time PCR reaction using Light Cycler® 96 Real-Time PCR (Roche Molecular Systems, Inc., Pleasanton, CA, USA). All reactions were performed in a volume of 14 µL. Each reaction solution consisted of 7 µL SYBR Green Master Mix (2x) (Amplicon, Odense, Denmark), 0.6 µL specific primers for MCM3AP-AS1 and β-actin (10 µM), 1 µL cDNA (100 ng/µL), and 5.4 L ddH_2O. The amplification conditions for LncRNA MCM3AP-AS1 gene replication were as follows: initial denaturation at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, which was followed by the final elongation at 72 °C for 5 min. Each sample was assessed twice, and β-actin was used as Housekeeping Gene Reference to normalize all gene expression data.

After completion of the Real-time PCR reaction, the cycle threshold (Ct) was measured for each sample. Ct was defined as the number of cycles that were required for the fluorescent intensity signal to cross the threshold. The Ct values obtained for each sample were entered into the Excel software. The Ct difference between the LncRNA MCM3AP-AS1 and β-actin was defined as ΔCt (ΔCt = Ct [lnc MCM3AP-AS1] – Ct [β-actin]). Then, the $2^{-\Delta Ct}$ was calculated to determine differences in lncRNA MCM3AP-AS1 expression levels in breast cancer tumour tissue as compared with adjacent non-tumour tissues, $x10^{-3}$.

Statistical analysis was performed using SPSS statistics version 24 and GraphPad Prism 7 software. A Mann–Whitney test was used to compare the gene expression levels between the two groups of breast cancer tumour tissue, and adjacent non-tumour tissues. Continuously variable data are presented as median with interquartile range (IQR). Receiver operating characteristic (ROC) curve analysis was performed to investigate the potential of lncRNA MCM3AP-AS1 as a diagnostic biomarker in breast cancer. In all analyses, the confidence interval of 95% and *P*-values <0.05 were regarded to be statistically significant.

Results

Relative lncRNA MCM3AP-AS1 expression in breast cancer tumour tissues (median/IQR 2.4 (0.01–8.4)) was higher as compared with adjacent non-tumour tissues (1.2 (0.01–5.6)) ($P < 0.001$) (Figure 1). There was no

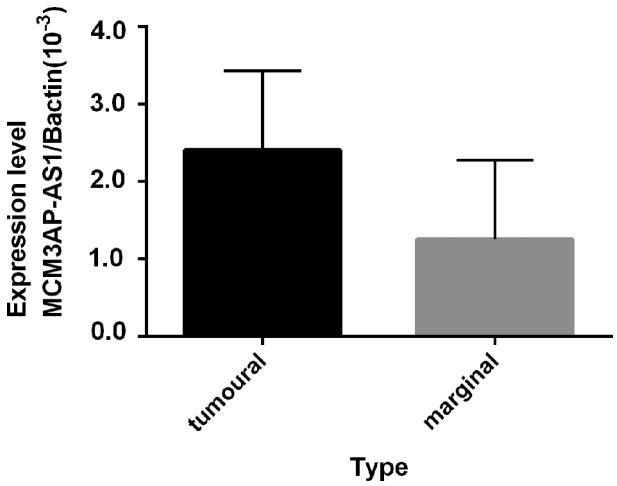

Figure 1. Expression of LncRNA MCM3AP-AS1 in breast cancer tumour tissue and marginal normal tissue. Data median (interquartile range).

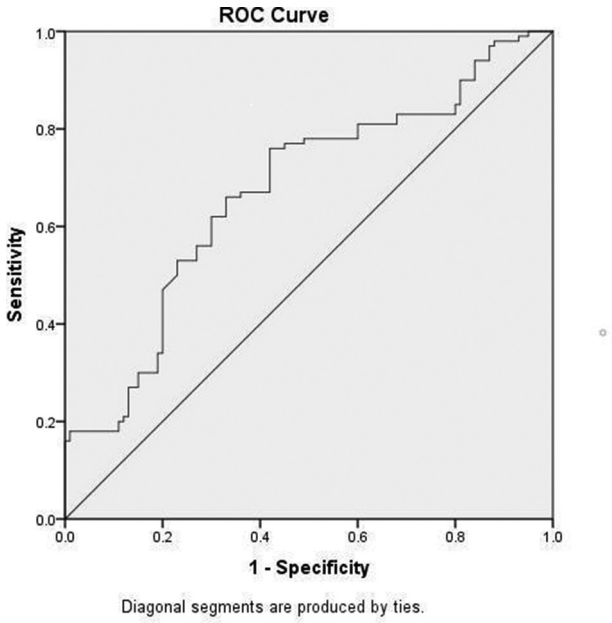

Figure 2. Receiver operating characteristic curve relating to LncRNA MCM3AP-AS1 gene expression levels.

Table 1. The association analysis between MCM3AP-AS1 gene expression level and the clinicopathological features of 102 breast cancer patients.

Characteristics	No. of patients	LncRNA MCM3AP-AS1 level	P-value
Age (years)			0.185
<45	48	2.4 (0.14–8.4)	
≥45	54	2.4 (0.01–8.3)	
Histology			0.806
Ductal carcinoma	69	2.3 (0.01–8.4)	
Lobular carcinoma	33	2.4 (0.21–5.6)	
Oestrogen receptor expression			0.023
Positive	41	2.4 (0.21–8.3)	
Negative	61	2.0 (0.01–8.4)	
Progesterone receptor expression			<0.001
Positive	38	2.9 (0.21–8.3)	
Negative	64	1.9 (0.01–8.4)	
HER2 expression			0.065
Positive	18	3.1 (1.4–7.7)	
Negative	84	2.3 (0.01–8.4)	

Data median and IQR fold difference tumour/normal tissue versus β-actin ($\times 10^{-3}$).

difference in expression in women aged <45 years versus those age 45 or more, or in ductal versus lobular cancer. Expression was higher in those whose tumours were oestrogen or progresterone receptor positive, but the difference according to HER2 expression was not significant (Table 1). The ROC curve analysis showed a maximum area under the curve of 0.67 (95% CI 0.60–0.75, $P < 0.001$) with a sensitivity and specificity of 58%, and 76%, respectively (Figure 2).

Discussion

Advances in the research of lncRNAs have indicated that lncRNAs are involved in major biological activities of cells and are associated with the pathology of many malignancies and so may be regarded as molecular diagnostic markers and treatment targets for various diseases [15]. Recently, studies have demonstrated that although lncRNAs usually have low rates of gene expression, aberrant regulation of lncRNA expression is associated with tumour formation in malignancies such as breast, colorectal, lung, and gastric cancer. Moreover, lncRNAs have been found that changes in their gene expression can be used as a marker to predict, diagnose, and treat breast cancer [20–24]. Antisense lncRNAs may be a contributing factor in inactivating the X-chromosome, imprinting the genome, and initiating and promoting some diseases [12].

Our data add to the literature of lncRNA MCM3AP-AS1 in cancer. Overexpression of MCM3AP-AS1 is also a feature of glioblastoma endothelial cells, and knockdown of MCM3AP-AS1 was associated with the ability of cells to survive, migrate, form tubes, and inhibit the glioblastoma angiogenesis *in vitro* [19]. Others suggest that, in hepatocellular carcinoma (HCC), lncRNA MCM3AP-AS1 can serve as an oncogene as increased expression level of MCM3AP-AS1 was significantly linked between the growth and progression of HCC tumours. Additionally, the findings have indicated that knockdown of MCM3AP-AS1 inhibited the promotion of HCC cell cycle, the proliferation of cells, the formation of colonies, and stimulation of the apoptosis *in vitro*. Moreover, the depletion of MCM3AP-AS1 had led to the suppression of HCC tumour development *in vivo* [25]. A study on papillary thyroid cancer has reported a high expression level of lncRNA MCM3AP-AS1, which could lead to the development and invasion of papillary thyroid tumours [26]. Studies addressing lung adenocarcinoma and non-small-cell lung carcinoma have found that MCM3AP-AS1 is up-regulated, but down-regulated in ovarian cancer tissues as compared to normal marginal tissues [27]. Another study examining lncRNA MCM3AP-AS1 in osteoarthritis has indicated that it is up-regulated and promoted

chondrocyte apoptosis by regulation of miR-142-3p and HMGB1 axis [28].

In studies on breast cancer, log-rank analysis of GEO data sets has indicated that MCM3AP-AS1 is one of the five lncRNAs that is related to the prognosis of breast cancer. It has been suggested that its overexpression correlates with the overall and poor relapse-free survival of breast cancer patients and that the up-regulation of MCM3AP-AS1 can be induced by oestrogen [29]. A further role for MCM3AP-AS1 was recently demonstrated by Chen et al. [30] who showed that it binds to miR028-5p, which in turn regulates the expression of centromere protein F, itself involed in cell cycling.

Our data represent an advance in biomedical science because it shows a significant overexpression of MCM3AP-AS1 in tumour tissues of breast cancer patients compared with adjacent non-tumour tissues, with greater expression in tumours positive for oestrogen and progesterone receptors.

Summary table

What is known about this subject:

- LncRNAs participate in various cellular biological functions and are associated with the pathology of many malignancies.
- Mis-regulation of LncRNAs expression is associated with tumour formation in breast cancer.
- Changes in MCM3AP-AS1 gene expression have been observed in several malignant cancerous tissues.

What this study adds:

- MCM3AP-AS1 expression is increased in breast cancer tissues compared to adjacent normal tissues
- Expression is increased in oestrogen and progesterone receptor-positive tissues

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Disclosure statement

The authors declare that there is no conflict of interest.

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