Short Communication

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The Study of Long Noncoding RNA, Meg3, Expression Level and Its Association with Clinicopathologic Features in Breast Cancer

E. Soleimanpour (MSc)[1], M.A. Hosseinpourfeizi (PhD)[1*], E. Babaei (PhD)[1], V. Montazeri (MD)[2]

1.Deptartment of Animal Biology, Faculty of Natural Sciences, University of Tabriz, Tabriz, I.R.Iran

2.Noornejat Hospital, Tabriz, I.R.Iran

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ABSTRACT

BACKGROUND AND OBJECTIVE: Breast cancer is the most common invasive cancer and the second main cause of cancer death in women. Long noncoding RNAs (lncRNAs) play a critical role in different cellular and molecular activities such as carcinogenesis. Maternally Expressed Gene 3 (MEG3), is a long noncoding RNA that deregulates in various types of cancers. The aim of this study is the evaluation of MEG3 expression level and its association with clinicopathological features of breast tumor tissues.

METHODS: In this case-control study, 40 fresh-frozen breast tumor specimens and their paired non-tumoral adjacent tissues were collected from breast cancer patients living in the northwestern region of Iran. All tumor samples belonged to the invasive ductal carcinoma. After RNA extraction and cDNA synthesis, the expression level of MEG3 in breast tumor tissues compared to the paired adjacent normal tissues was investigated using specific primers and quantitative real-time PCR (qRT-PCR). β2m was also used as an internal control for normalization.

FINDINGS: MEG3 expression level in all tumor samples significantly downregulated compared to the paired adjacent nontumoral specimens, with an average fold decrease of 3.355 (p=0.042). Low level of MEG3 in tumor tissues was also related to the age of patients (p=0.007), stage III (p=0.049) and lymph node metastasis (p=0.018).

CONCLUSION: The expression level of MEG3 significantly decreased in breast cancer and this downregulation was related to malignancy state of the tumor.

KEY WORDS: *Breast Neoplasms, Long noncoding RNAs, Biomarker, Lymph node*.

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***Corresponding author: M.A. Hosseinpourfeizi (PhD)**
Address Deptartment of Animal Biology, Faculty of Natural Sciences, University of Tabriz, Tabriz, I.R.Iran
Tel: +98 41 33362280
E-mail: pourfeizi@eastp.ir

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Introduction

Breast cancer is a heterogeneous disease and is one of the most common causes of death in women. Contrary to recent advances in diagnostic and prognostic methods of breast cancer such as: MRI scan, mammography, ultrasound of the breast, and the evaluation of protein markers, early diagnosis of cancer due to the inaccuracy, sensitivity and specificity of the mentioned methods has encountered with significant problems. So, in recent years, there have been many efforts to find high and early diagnostic biomarkers (1, 2).

According to studies conducted in recent years, over 90% of genomic DNA is transcribed, of which only 1 to 2% of the transcripts are translated into proteins, the rest being classified as non-coding RNAs. These non-coding regions of the genome include a wide variety of regulatory RNAs that have different roles in biogenesis, properties, and function, and are categorized into two categories: short non-coding RNAs, such as miRNA (8-22 Nucleotides) and high non-coding RNA (lncRNA) (more than 200 nucleotides) (3). MEG3 (Maternally Expressed Gene 3) is a non-coding transcript with a length of 1.6 kb in human, that transcribed from the MEG3 gene in DLK1 / MEG3 locus and the 32q14 chromosomal position. MEG3 is expressed as normal in many tissues (4), but its expression in the brain and pituitary gland is significantly higher (5).

MEG3 consists of ten exons, and up to today its 12 isoforms have been identified. These isoforms are called MEG3, MEG3a to MEG3k. The dominant isoform is MEG3, which contains exons 1-4 and 8-10 (6). Recent studies have shown that the expression of MEG3 in various types of cancers decreases and acts as a tumor suppressor. In cervical cancer, the reduction of MEG3 expression by affecting cell growth and apoptosis and targeting miR-21 leads to tumor progression (7). In the gastrointestinal tract cancers, MEG3 expression has been reduced in patients and related cell lines, which is associated with malignancy and tumor invasion (8).

In bladder cancer, MEG3 expression was significantly reduced in tumor samples, and this reduction was associated with increased autophagy and increased cellular growth (9). The results of the Sun and colleagues study indicated that MEG3 expression was reduced in breast tumor samples compared to the adjacent normal tissue of the tumor. In order to investigate the effects of MEG3 on MCF-7 and MB231 breast cancer cells, increased the expression of MEG3 in these cell lines erratically, and it was observed that increasing in its expression was associated with decreasing the ability of cell proliferation, colony formation, immigration and invasion. It was also reported that MEG3 affects these processes by increasing the activity of P53 and subsequently affecting its target genes, such as P21, Mapsin and KAI1 (10).

Studies have shown that only a complete transcript of MEG3 has the ability to activate P53 (11). Zhang et al. reported that the expression of MEG3 in breast cancer declined, which is due to the effect on the AKT signaling pathway, which is a vital pathway for the growth and angiogenesis of the breast cells, leading to increased cell growth and proliferation, angiogenesis, metastasis and invasion (12).

Regarding the functional role of MEG3 in the progression of various cancers as well as extensive studies on the role and importance of non-coding RNAs in the incidence of malignancies, the purpose of this study was to investigate the expression of MEG3 in invasive breast tumor samples of the tubes in comparison with the normal marginal samples of the same tumor, the focus is on the possibility of using it as a diagnostic or prognostic biomarker and studying its relevance to clinicopathologic characteristics for the first time in Iran.

Methods

This case-control study was approved by the ethical committee of medical research of Noor Nejat Tabriz Hospital with an ethical code of 3259/4/5, as well as written and informed consent of breast cancer patients while respecting ethical principles and maintaining confidential patients information on tumor tissue and nontumoral tissue of tumor margin of 40 women with breast cancer referred to Noor-e-Nejat hospital in Tabriz. Tumor margins were used as control samples. The tissue samples were freshly prepared from the surgery room.

All selected samples belonged to the invasive ductal carcinoma after pathological examination, and the patients had not undergone any chemotherapy or radiotherapy prior to sampling. The mean age of the patients was 50±2.03 (35-80 years old) and all patients were similar in terms of gender, place of residence and lifestyle. Since the age, tumor size, degree of differentiation and the rate of tumor metastasis to the

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lymph nodes are important factors in the onset and progression of the tumor, as well as selection of appropriate treatment method, tumor samples were categorized due to these factors.

RNA extraction was performed using a TRIzol extraction solution (Invitrogen, Carlsbad, CA, USA, and Lot No. 5017511 according to the factory's instructions) (13, 14). 1 milliliter of TRIzol solution was added to the homogenized sample and incubated for 15 minutes at room temperature. Then, 200 µl of chloroform was added and after incubation at room temperature for 15-20 minutes, centrifuged at 12000 g at 4 ° C for 20 minutes. After centrifugation, the higher aqueous phase was transferred to another micro tube and the 100% isopropanol was added (as same volume of aqueous phase).

Following 10-minute incubation at room temperature and a 15-minute centrifuge with 12000 g rounds, the aqueous phase was discarded and the resulting precipitate was washed with 1 milliliter of 75% ethanol added and re-centrifuged with 7500 g. The aqueous phase was discarded and the precipitate was dissolved in RNase-free water for further testing and stored until use at -80 °C.

The quality and quantity of RNAs extracted using agarose gel and 260 to 280 nm RNA adsorption were measured using a nanodrop machine. To eliminate possible contamination with DNA, the RNA was treated by the DNase I enzyme prior to the RT-PCR reaction. The treated RNA with DNase I was incubated for 5 minutes at 70 ° with 0.5 µl of Random Hexamer enzyme. In the next step, 1 µl of dNTP mixture and 2 µl of RT buffer were added to the required amount of sterilized distilled water and incubated for 5 minutes at 37 °C and finally, 0.5 µl of RT enzyme was added. The reaction mixture was incubated at 42 °C for 1 hour and 20 minutes.

β2m gene is a housekeeping gene and is considered as an internal control gene. Primers used in this method were designed and evaluated using the GeneRunner software (version 4) and the OLIGO software (version 5.7). Specificity of primers was verified in terms of secondary structures by the NCBI site as well as reproduction sequences by the UNAfold site (15). The primer patterns were described in Table 1. After cDNA synthesis, quantitative MEG3 expression was performed using qRT-PCR method using SYBR® Green Supermix (Takara, Japan, Lot No. AF31017N). The reaction was carried out in 40 cycles, denaturation at temperature of 94 ° C for 25 seconds, the annealing at 59 °C for 30 seconds, and a 25 second expansion step at 72 °C.

In order to investigate the relationship between MEG3 expression changes with tumor size, the tumor samples were divided in two groups smaller than 2.5 cm (50%) and larger than or equal to 2.5 cm (47.5%). Tumors were classified into positive (52.5%) and negative (47.5%) groups for lymph node involvement. The negative group means the absence of cancer cells and the positive group means the presence of cancer cells in the lymph nodes.

Tumor samples are divided into three categories I, II and III (16) based on the TNM system (tumor size-the number of lymph nodes involved-metastasis), according to the differentiation degree. In the present study, tumors with differentiation degree of I and II were classified in one group (57.5%) and invasive tumors with differentiation degree of III (42.5%) were classified in another group. Data from expression changes using the 2-ΔΔCt method and using REST software were analyzed to determine the rate of expression changes in tumor samples compared to normal tumor marginal samples.

Other statistical analyzes of the clinicopathologic characteristics of tumor samples were analyzed using SPSS software (version 16) and T-test. $P<0.05$ was considered significant. Also, SPSS software was used to check the diagnostic accuracy, biomarker power and MEG3 sensitivity and specificity for drawing ROC curve. As the perimeter of ROC curve is closer to one, the gene under consideration will have a higher diagnostic accuracy (17, 18).

Table1. Primer sequence used for MEG3 and β2m

Gene name		Primer sequence	Product length(bp)
MEG3	Forward	5'-TGCCCATCTACACCTCACGA-3'	113
	Reverse	5'- GTCCTCTTCATCCTTTGCCAT-3'	
β2m	Forward	5'- CTACTCTCTCTTTCTGGCCTG-3'	191
	Reverse	5'- GACAAGTCTGAATGCTCCAC-3'	

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Results

The changes in MEG3 non-coding RNA expression in tumor tissue samples (40 samples) compared with normal tumor margin (40 samples) showed a reduction of 355.3 times in tumor samples, which showed a 95% confidence interval, p=0.042, It was reported that a significant decrease in expression of MEG3 in tumor samples compared to the normal tumor margin samples (Fig 1a). There was no significant relationship between decreased MEG3 expression and tumor size. There was a significant relationship between the reduction of MEG3 expression and lymph node involvement (Fig 1b). A significant relationship was found between decreasing MEG3 expression and tumor invasive intensity (p=0.049) (Fig 1c). Age is one of the most important risk factors for breast cancer. In terms of age, patients were classified into two age groups less than 50 years old (45%) and older than 50 years (55%). The results showed that there was a significant relationship between MEG3 expression decrease with age of patients (p=0.007) (Fig 1d) (Table 2). In order to determine the MEG3 diagnostic potential, as a specific biomarker for diagnosis of breast cancer a ROC curve was drawn. The AUC for MEG3 was calculated to be 0.67 (p=0.037), which indicates that MEG3 has sensitivity and specificity for use as a diagnostic biomarker (Fig 2).

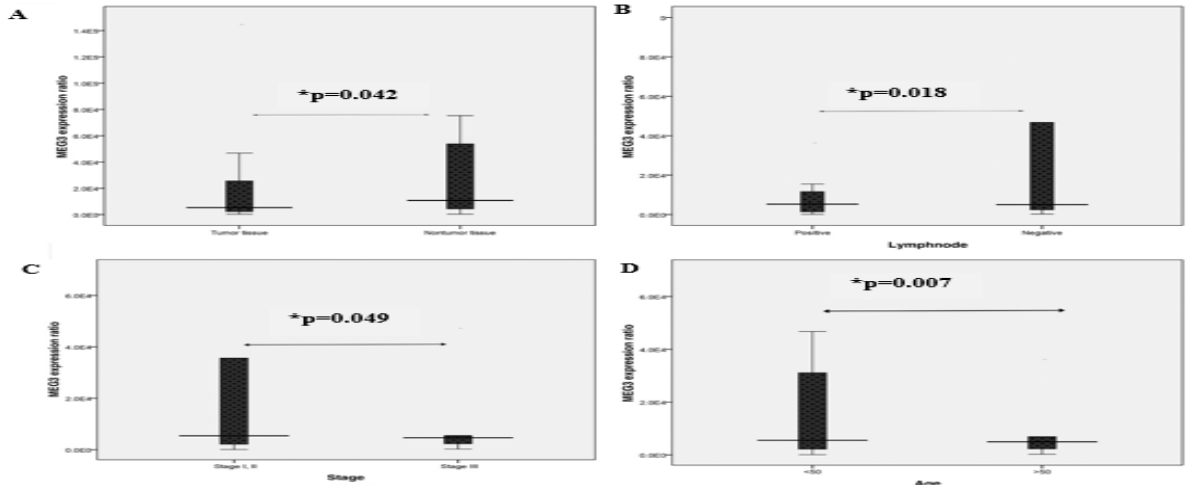

Figure 1. Investigating the difference between MEG3 expression and clinico-pathological characteristics of Breast tumor. A) MEG3 in breast tumor samples showed a significant decrease compared to normal tumor marginal samples. It also has a direct and meaningful relationship with reduces the expression b) metastasis to the lymph nodes, c) a high degree of differentiation of the tumor, and d) high age of patients. * The indicator is significant and P<0.05

Table 2. Clinico-pathological number and characteristics of breast tumor samples

Variables	samples N(%)	P-value
Tumor tissue	40(100)	0.042*
Tumor margin tissue	40(100)	
The size of the tumor		
Larger and equal to 2.5 centimeters	19(47.5)	0.059
Smaller than 2.5 centimeters	20(50)	
Degree of TNM distinction		
**Stage I,II	23(57.5)	0.049*
Stage III	17(42.5)	
Lymph node metastasis		
Positive	21(52.5)	0.018*
Negative	19(47.5)	
Age		
More than 50 years old	22(55)	0.007*
Less than 50 years	18(45)	

****TNM=tumor-node-metastasis staging * Indicates significant and P<0.05**

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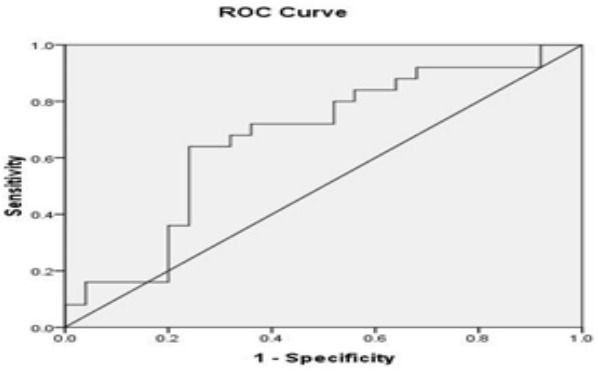

Figure2. ROC curve for MEG3 to verify the biomarker potential of MEG3. The area under curve (AUC=0.67) indicates the sensitivity and specificity of MEG3 for use as a diagnostic biomarker for breast cancer

Discussion

The present study to investigate changes in MEG3 expression level showed the expression reduction in breast tumor samples in tumors than normal tumor margins. By examining 257 breasts tissue samples with qRT-PCR, Shi and colleagues showed that expression of MEG3 in breast tumors was reduced, and this reduction was associated with metastasis to lymph nodes and degree of tumor differentiation (19). Another study showed that MEG3 expression in breast cancer was low, and this reduction in expression led to cellular malignancy (10).

Along with the results of this study, Zhang and colleagues reported that cervical cancer decreases MEG3 expression, which affects cell proliferation and apoptosis by regulating expression of miR-21. This has shown that MEG3 can be considered as a therapeutic goal in cervical cancer (7). In another study, they showed that this decrease in MEG3 expression was due to hyperemethylation of the MEG3 promoter, and low levels of MEG3 were associated with recurrence of disease and short-term patient survival (20).

In studies in lung cancer, Lu and colleagues reported that MEG3 expression in cancerous tissues was reduced to normal tissues, and this reduction was related to the degree of tumor differentiation and tumor size (21). In addition, they showed that the increased expression of MEG3 not only reduced the proliferation of NSCLCs and induced apoptosis but also inhibited tumorigenicity by increasing the expression level of P53 and MDM2 (21). Also, in the gastrointestinal cancer, a decrease in MEG3 was observed, and this low expression level with an effect on Bcl-2 and miR-181 caused cell proliferation, migration, and invasion of cancer cells (22). Decreased expression of MEG3 has also been reported in other cancers such as bladder cancer (9), glioma (23), liver cancer (24), colorectal cancer (25), and osteosarcoma (26). In the present study, there was a significant relationship between the reduction of MEG3 expression with metastasis of lymph nodes and malignancy or a high degree of tumor differentiation in breast cancer. These findings were confirmed by studies by Zhang et al. in cervical cancer (7) and Shi et al. in breast cancer (19).

But, contrary to findings from cervical cancer (7) and lung cancer (21) that there was a significant relationship between MEG3 reduction and tumor increase, there was no significant relationship in breast cancer, which may be due to differences in the number of samples or the size of the tumors under study. In this study, there was also a significant relationship between age increase and MEG3 expression declines, so far no similar report has been presented in this regard, and could be seen as a key factor in increasing age in the incidence and progression of cancer. In addition, the ROC curve analysis showed that MEG3 had an appropriate sensitivity and specificity for use as diagnostic biomarker for breast cancer, which is consistent with findings from cervical (20) and ovaries cancers (28).

According to the data from the ROC curve, and with reference to the effect of MEG3 on the progression and malignancy of the tumor, MEG3 can be considered as a diagnostic marker, prognosis and also a therapeutic target for cancer. In general, the findings of this study showed that MEG3 expression in breast tumor samples decreases, and this reduction in expression plays an important role in the progression and malignancy of the tumor.

Considering the limitations of the present study, such as the limited statistical population, studying in a larger population, based on the molecular mechanism of MEG3 function, is recommended for use as a therapeutic goal.

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