Expression levels of breast cancer-related GAS5 and LSINCT5 lncRNAs in cancer-free breast tissue: Molecular associations with age at menarche and obesity

Yaser Mansoori PhD1,2 | Mohammad Bagher Tabei PhD3 | Alireza Askari MD4,5 | Pantea Izadi PhD1 | Abdolreza Daraei PhD6 | Milad Bastami PhD7,8 | Mohammad Mehdi Naghizadeh PhD2 | Ziba Nariman-Saleh-Fam PhD9 | Behnam Mansoori MD2 | Javad Tavakkoly-Bazzaz MD, PhD1

1Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
2Noncommunicable Diseases Research Center, Fasa University of Medical Sciences, Fasa, Iran
3Department of Medical Genetics, School of Medical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
4Department of Orthopedy, Shiraz University of Medical Sciences, Shiraz, Iran
5Bone and Joint Reconstruction Research Center, Shafa Orthopedic Hospital, Iran University of Medical Sciences, Tehran, Iran
6Department of Genetics, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran
7Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
8Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
9Women’s Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

Correspondence
Javad Tavakkoly-Bazzaz, MD, PhD, Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Email: tavakkolybazzaz@tums.ac.ir

Abstract
Long noncoding RNAs (lncRNAs) constitute a major class of the human transcriptome which play crucial roles in the key biological processes of both normal and malignant breast cells. Although the aberrant expression of lncRNAs has been well-documented in breast cancer (BC), little is currently known about the association between their expression levels in the breast tissue of healthy women and BC risk factors, especially the reproductive or demographic characteristics that are among the most well-known BC risk modifiers. This study was an attempt to investigate the correlation between the expression levels of 2 breast cancer-related lncRNAs, including GAS5 and LSINCT5, and reproductive and demographic characteristics in 145 normal breast tissues that were obtained from women without breast cancer undergoing cosmetic surgery. Total RNA was extracted from fresh normal breast tissues, and the expression level of target lncRNAs was quantified using real-time qPCR. Differences in the mean normalized gene expression among the subgroups of different variables were analyzed. The expression levels of both genes was lower in the overweight-obese (BMI ≥ 25) subgroup than that in the normal BMI (BMI < 25) subgroup (GAS5 P = .019, LSINCT5 P = .036). Moreover, the expression level of GAS5 was negatively correlated with BMI (r: −.170, P: .041). The expression level of GAS5 was higher in women with late menarche (>13 years) than that with early menarche (≤13 years; P = .017). These findings may assist to obtain insights into the molecular mechanisms through which the reproductive or obesity-related estrogen changes contribute to the breast carcinogenesis. In conclusion, this study presents the first evidence for the presence of a link between the lncRNA expression and the reproductive or obesity related factors in the breast tissue of healthy women.

KEYWORDS
breast, expression, lncRNA, reproductive factor
INTRODUCTION

Noncoding RNAs (ncRNAs) are functional RNA molecules involved in regulation of gene expression at the transcriptional and post-transcriptional levels. They are usually divided into 2 broad categories: short ncRNAs (eg microRNAs or miRNAs) and long ncRNAs (or lncRNA). miRNAs are about 22 nucleotides in length and usually bind to specific regions at the 3’-untranslated region of target messengers by forming a short complementary sequence, leading to the degeneration of the messenger transcript or blockage of translation. Each miRNA can modulate the expression of several target genes that are involved in a variety of cellular processes and thereby play important roles in key biological pathways both in normal physiology and in disease.

They regulate nearly all cellular processes altered during tumorigenesis. In the other side, lncRNAs are a diverse class of regulatory RNAs with more than 200 nucleotides in length. They can be located within introns, between genes or be transcribed as natural antisense of coding genes. According to the position and direction of transcription with respect to other genes, lncRNAs are classified into subtypes such as antisense, intergenic, overlapping, intronic, bidirectional, and processed. They can be expression in a cell, tissue, or developmental stage-specific manner. LncRNAs constitute a major class of the human transcriptome. Although a large number of lncRNAs have remained functionally unannotated, the emerging evidence suggests that some of them may play crucial roles in key biological pathways.

For example, the evaluation of the lncRNA landscape of breast cancer (BC) has revealed a wide range of biological functions for lncRNAs and suggests that data about the lncRNA expression profile may not only advance our understanding of tumor biology but also is of the diagnostic and prognostic value. Interactions of various environmental and genetic alterations have contributed to the complexity of BC pathogenesis. Therefore, understanding the mechanisms by means of which lncRNAs may contribute to the BC development may have great implications for the disease biology.

The growth arrest-specific 5 (GASS) and long stress-induced non-coding transcript 5 (LSINCT5) lncRNAs are of particular interest as they regulate cell proliferation. GASS is recognized as a well-known tumor suppressor lncRNA in many types of cancers, including BC. Its tumor suppressor activity is attributed to both the inhibition of cell proliferation and the promotion of apoptosis. GASS is downregulated and associated with poor survival in BC. On the other hand, LSINCT5 is a stress-induced lncRNA that promotes cell proliferation in breast tissues and is upregulated in BC cell lines and tumor tissues.

Although the aberrant expression of such lncRNAs has been reported in the breast tumor tissues, little information is currently known about their expression status in the breast tissue of healthy women and its potential relation to the BC risk factors. A number of hormone-related reproductive or demographic characteristics, including early menarche, nulliparity, lack of breast feeding, late menopause, and obesity are among the well acknowledged risk factors for BC. Given that lncRNAs are involved in the regulation of such processes as reproduction and obesity, it is speculated that lncRNAs may be related to such risk factors. This study investigated the expression levels of GASS and LSINCT5 lncRNAs in normal breast tissues and their potential correlation with the reproductive and demographic characteristics.

MATERIALS AND METHODS

Study population

The subjects of the current study consisted of 145 healthy women who had undergone cosmetic mammoplasty between 2013 and 2016 at 3 different centers including the Vali-e-Asr Hospital, the Sohrevardi Surgery Center and the Mehr-e-Sina Surgical Center. The participants of the study had no personal or family history of breast cancer or any other types of cancers. An informed consent regarding the authorized use of each participant’s specimens, and clinical information was obtained from each participant in the current study. In addition, the demographic and reproductive characteristics were recorded using a questionnaire. Table 1 shows the characteristics of the study participants. In terms of age, the participants were 19-70 years old (mean ± SD: 38.81 ± 10.09). Eighty-five (~58%) participants were aged <40 years, and 60 (~42%) were ≥40 years old. Among the 145 participants, 113 (~80%) were parous (P) and 32 (~20%) were nulliparous. Body mass index (BMI, kg/m²) subgroups were defined as follows: normal, 18.5 ≤ BMI < 25; overweight-obese, BMI ≥ 25. Regarding the menopausal status, 124 (~85%) participants were at premenopausal and 21 (~15%) were in postmenopausal status. Those participants at the postmenopausal state were categorized based on the menopause age of <50 or ≥50 years. Moreover, the participants were divided into 2 subgroups based on the age at menarche of <14 or ≥14 years. The age at the first full-term pregnancy and breast feeding duration were also recorded. Parous participants were divided into 2 subgroups according to the age at the first full-term pregnancy of <25 or ≥25 years.

Breast tissue sampling and RNA extraction

Total RNA was extracted from fresh normal breast tissue using the RiboEx solution (GeneAll, South Korea) according to the manufacturer’s instructions. For removal of the DNA contamination, the total RNA was treated using DNase (Catalog number: 2270A, Clontech, Japan) according to the manufacturer’s instruction.

Reverse transcription and real-time PCR

The PrimeScript kit (Clontech, Japan) was used for cDNA synthesis. A 10 µL cDNA synthesis reaction included 500 ng of total RNA,
0.5 μL of PrimeScript RT enzyme mix (200 U/μL), 2 μL of 5× PrimeScript Buffer, 0.25 μL of RNase Inhibitor (40 U/μL), 0.5 μL of oligo dT Primer (50 μmol/L), 0.5 μL of random hexamer (100 μmol/L) and RNase free dH₂O. Real-time PCR reactions were performed on RotorGene 6000 (QIAGEN, Germany) using SYBR Premix Ex Taq II (Clontech, Japan) and specific primers. A 10 μL real-time PCR reaction included 1 μL of cDNA, 5 μL of SYBR Premix Ex Taq II master mix, 0.5 μL of specific forward primer (5 μmol/L), 0.5 μL of specific reverse primer (5 μmol/L), and 3 μL DNase-free dH₂O.

The sequence of primers was as follows. GAS5: TGGTTCTGCTCTGGTAACG (forward) and AGGATAACAGGTTACCTGC (reverse); LSINCT5: GGACCTGCAAAGTACCCATAGGCA (forward) and GCTGTCTCCTCCAGCTCCAAAG (reverse); B2M: AGATGAGTATGCCTGCCGTG (forward); and GCGGCATCTTCAAACCTCCA (reverse). The expression of GAS5 and LSINCT5 was normalized to B2M housekeeping gene. Efficiency-corrected gene expression value was calculated for each sample gene. The efficiency of reactions was evaluated by amplifying the twofold dilution series.

### TABLE 1 Characteristics of the study population and the results of GAS5 and LSINCT5 expression levels in the variable subgroups by categorical analyses

| Characteristics            | N  | GAS5 Mean | GAS5 SD | P*  | LSINCT5 Mean | LSINCT5 SD | P** |
|----------------------------|----|-----------|---------|-----|--------------|------------|-----|
| Parity                     |    |           |         |     |              |            |     |
| Parous                     | 113| 0.1214    | 0.1012  | .469| 0.4911       | 0.6606     | .797|
| Nulliparous                | 32 | 0.1371    | 0.1305  |     | 0.5817       | 0.8820     |     |
| Age (y)                    |    |           |         |     |              |            |     |
| <40                        | 85 | 0.1302    | 0.1085  | .478| 0.5329       | 0.8373     | .887|
| ≥40                        | 60 | 0.1172    | 0.1079  |     | 0.4801       | 0.4907     |     |
| BMI (kg/m²)                |    |           |         |     |              |            |     |
| 18.5 ≤ BMI<25              | 51 | 0.1533    | 0.1069  | .019| 0.7268       | 1.0103     | .036|
| ≥25                        | 94 | 0.1094    | 0.1061  |     | 0.3941       | 0.4459     |     |
| Age at menarche (y)        |    |           |         |     |              |            |     |
| <14                        | 98 | 0.1101    | 0.0886  | .017| 0.5237       | 0.8172     | .355|
| ≥14                        | 47 | 0.1556    | 0.1363  |     | 0.4847       | 0.4266     |     |
| Age at menopause (y)       |    |           |         |     |              |            |     |
| <50                        | 14 | 0.1357    | 0.1177  | .175| 0.5397       | 0.4451     | .520|
| ≥50                        | 10 | 0.0789    | 0.0578  |     | 0.3648       | 0.4476     |     |
| Menopausal status          |    |           |         |     |              |            |     |
| Pre                        | 121| 0.1274    | 0.1098  | .527| 0.5199       | 0.7560     | .911|
| Post                       | 24 | 0.1121    | 0.0998  |     | 0.4669       | 0.4451     |     |
| Number of full term pregnancies |     |           |         |     |              |            |     |
| 0                          | 32 | 0.1336    | 0.1300  | .811| 0.5655       | 0.8730     | .709|
| 1-2                        | 82 | 0.1199    | 0.0881  |     | 0.5325       | 0.7326     |     |
| ≥3                         | 31 | 0.1285    | 0.1310  |     | 0.3973       | 0.4213     |     |
| Age at FFTP (y)            |    |           |         |     |              |            |     |
| <25                        | 80 | 0.1195    | 0.1084  | .756| 0.4964       | 0.7351     | .243|
| ≥25                        | 33 | 0.1260    | 0.0825  |     | 0.4781       | 0.4394     |     |
| Abortion                   |    |           |         |     |              |            |     |
| No                         | 113| 0.1252    | 0.1028  | .940| 0.5539       | 0.7727     | .350|
| Yes                        | 32 | 0.1236    | 0.1261  |     | 0.3657       | 0.4336     |     |
| Breast feeding (mo)        |    |           |         |     |              |            |     |
| No                         | 40 | 0.1316    | 0.1215  | .642| 0.6588       | 1.0204     | .496|
| Yes                        | 105| 0.1223    | 0.1030  |     | 0.4548       | 0.5493     |     |

*Number of participants.

*P value of the parametric tests.

**P value of the nonparametric tests.

Bold values show the statistically significant levels (p < 0.05).

BMI, body mass index; FFTP, first full-term pregnancy; SD, standard deviation.
of the pooled cDNA (ie, a mixture of all cDNAs) and evaluating the slope of the corresponding standard curve. The serial dilution reactions were performed in triplicate and all other reactions, including the no template control (NTC), in duplicate. The standard curves and the corresponding efficiencies for GAS5, LSINCT5, and B2M are depicted in Figure 1.

### 2.4 Statistical analysis

The data were presented by mean and standard deviation. According to Kolmogorov-Smirnov test, the normal distribution of GAS5 was met but the distribution of LSINCT5 was departed from the normality assumption. Therefore, the comparison of GAS5 among the subgroups was made using t test or ANOVA. The relationship of GAS5 expression with quantitative variables was analyzed by Pearson's correlation coefficient. Then, the expression level of LSINCT5 was compared between the subgroups through nonparametric tests like Mann-Whitney and Kruskal-Wallis. Spearman correlation coefficient was used for LSINCT5. All the statistical analyses were performed in IBM SPSS statistics version 21 (IBM SPSS Inc, Chicago, IL, USA). The P-value of <.05 was considered as the level of significance.

### 3 RESULTS

#### 3.1 Expression of GAS5 and reproductive/demographic characteristics

The t test revealed that the expression level of GAS5 was significantly higher in the subgroup of participants with the age at menarche of ≥14 years than that in those with the age at menarche of <14 years (P: .017, Figure 2A). Additionally, the expression level of GAS5 was significantly lower in participants in the overweight-obese subgroup than that in participants in the normal BMI subgroup (P: .019, Figure 2B). For the other variables, no significant difference was identified in the expression level of GAS5 among the subgroups.

Moreover, the Pearson correlation analysis showed that the expression level of GAS5 was negatively correlated with BMI (r: -.170, P: .041). The Pearson analysis did not show any significant correlation between the expression level of GAS5 and the other studied variables (Table 2).

#### 3.2 Expression of LSINCT5 and reproductive/demographic characteristics

The expression level of LSINCT5 was significantly lower among the participants in the overweight obese subgroup than that among the participants in the normal BMI subgroup (P: .036, Figure 2C). For the other variables, no significant difference was found in the expression level of LSINCT5 among the subgroups. Furthermore, Spearman's analysis did not represent any significant correlation between the expression level of LSINCT5 and the studied variables (Table 2).

### 4 CONCLUSION

A large number of lncRNAs have been recognized to be involved in various stages of reproductive development, hormone response, obesity, as well as BC development. Given the diverse roles lncRNAs play in these biological processes, it is not a surprise that lncRNAs may also be, at least in part, related to some hormone-related BC risk factors such as early menarche, late first full-term pregnancy, late menopause, nulliparity, and obesity. It is well established that these factors contribute to the risk of BC; however, the exact molecular mechanisms through which they influence the risk of BC are not yet understood. The identification of early molecular changes in the breast tissues that are mediated by or related to the hormonal risk factors may help to gain an insight into the underpinning mechanisms through which such factors contribute to BC development. Such an insight may pave the way for the development of appropriate preventive approaches. In this study, it was
hypothesized that the expression level of lncRNAs in breast tissues of healthy individuals may be correlated to the reproductive and demographic factors.

Increasing evidence suggests that GAS5 and LSINCT5 play important roles in carcinogenesis. A number of studies have documented the contribution of GAS5 to the progression of different cancers, including ovarian, lung, renal cell, and especially breast carcinoma. GAS5 acts as a decoy glucocorticoid response element and sensitizes cells to apoptosis by suppressing the glucocorticoid-mediated induction of several target genes. In other words, it is considered as a ribo-repressor of the glucocorticoid receptor that regulates the cell survival and metabolic activities.

Recently, a ceRNA (ie, competing endogenous RNA) role for GAS5 has been identified in HER2-positive BC. It has been shown that GAS5 acts as a molecular sponge for miR-21 that leads to the derepression of a miR-21 target gene (ie, PTEN) and, thereby, suppresses cancer proliferation. The downregulation of GAS5 is associated with cancer in breast as well as other tissues and reflects its potential tumor suppressor role.

GAS5 expression has implications for chemotherapy in BC. It has been shown that the reduced GAS5 expression attenuates the apoptosis induction by classical chemotherapeutic agents, and, thereby, adversely affects patient prognosis. Furthermore, the downregulation of GAS5 causes trastuzumab resistance in HER2-positive BC; therefore, it is considered as a novel prognostic marker and candidate drug target for this type of BC. On the other hand, LSINCT5 is a stress-induced lncRNA overexpressed in breast and ovarian cancer cell lines and tumor tissues that affects cellular proliferation.

**TABLE 2** Correlation of GAS5 and LSINCT5 lncRNAs expression with the reproductive and demographic variables

| Characteristics | GAS5 (Pearson correlation) | LSINCT5 (Spearman correlation) |
|-----------------|---------------------------|--------------------------------|
| Age (y)         | r = −.120                 | r = −.048                       |
|                 | P = .152                  | P = .566                        |
| BMI (kg/m²)     | r = −.170                 | r = −.118                       |
|                 | P = .041                  | P = .157                        |
| Age at menarche (y) | r = .102                | r = .012                        |
|                 | P = .224                  | P = .887                        |
| Age at menopause (y) | r = .118                | r = −.005                       |
|                 | P = .611                  | P = .982                        |
| Breastfeeding duration (mo) | r = −.017           | r = −.114                       |
|                 | P = .859                  | P = .228                        |
| Age at FFTP     | r = −.031                 | r = .040                        |
|                 | P = .748                  | P = .671                        |

Bold values show the statistically significant levels (p < 0.05).

FFTP, first full-term pregnancy.

**FIGURE 2** GAS5 expression in the subgroups of the age at menarche (A) and BMI (B) variables and LSINCT5 expression in the subgroups of BMI variable (C). The vertical axes represent mean efficiency-corrected B2M-normalized expression levels. Error bars represent standard error of the mean.
NEAT-1 and a protein coding gene PSPC1.\textsuperscript{15} It also exhibits an oncogenic role and predicts a negative prognosis in gastric cancer.\textsuperscript{26} In spite of the availability of the above-mentioned evidence for the involvement of the 2 lncRNAs in carcinogenesis, few studies, if any, have been carried out to scrutinize the correlation of the expression status of the lncRNAs in healthy breast tissues with the reproductive and demographic parameters.

The categorical analysis of the current study revealed that both GAS5 and LSINCT5 were downregulated in the overweight-obese subgroup compared to the normal BMI subgroup (Table 1). This study also revealed that the expression level of GAS5 was higher among the participants with late menarche than those with early menarche. The relationship between obesity and BC is complex. In postmenopausal women, high BMI has been recognized as a risk factor for BC.\textsuperscript{17} In premenopausal women, however, there is no universal consensus. Although some studies suggest that BMI has no significant effect on the incidence of BC during premenopausal period,\textsuperscript{30} recent findings indicate that considering body size over the life-course may point to a more clearly characterized relationship and clarify inconsistent findings across studies.\textsuperscript{31} In this setting, premenopausal BC is influenced by adolescent, but not adult, body size with greater body mass associated with a reduction in BC risk.\textsuperscript{31} A possible explanation for the correlation of BMI and BC risk is that high BMI is associated with an increased level of circulatory estrogen, and, in combination with a decreased circulation of sex hormone binding globulin, may lead to a greater tissue availability of estrogen.\textsuperscript{18,32} On the other hand, the increased risk of BC associated with early menarche is also attributed, at least in part, to the lifetime exposure to estrogen.\textsuperscript{33} It has been shown that estrogen is linked to the regulation of lncRNAs in BC. Recent single molecule sequencing approaches have shown numerous lncRNA dysregulations that are among the most clinically relevant estrogen-induced changes and may be of prognostic significance in relation to BC survival.\textsuperscript{34} Similarly, a great number of estrogen-responsive lncRNAs that regulate cell proliferation and growth factor signaling pathways have been identified.\textsuperscript{35} Nevertheless, the possible regulation of GAS5 and LSINCT5 through the estrogen pathway is unknown and awaits future studies. Of note, the present study lacks a positive control such as hormone receptor-positive BC patients, and therefore, further studies are needed to determine whether the observed correlations of the lncRNAs with BMI and age at menarche are relevant to the estrogen pathway. Given the influence of high BMI on BC risk, the negative correlation of the GAS5 expression with BMI is consistent with its tumor suppressor activity in BC, implying that this correlation may be relevant to BC. In contrast, LSINCT5 has oncogenic activity in BC. Therefore, the finding that LSINCT5 is expressed to lower levels in the overweight individuals may contradict with its oncogenic role in BC. It should be noted that the present study did not consider a matched BC group and further studies are needed to evaluate the relevance of the observed correlations with the BC development. Moreover, most participants in the present work were in premenopausal status. Future studies may benefit from comparing premenopausal and postmenopausal participants, considering the circulatory levels of the estradiol and selecting a more uniform age range for the study population. Given the profound effects of the pregnancy on the breast tissue gene expression, taking the timing from the last postpartum for parous women into account may greatly help to decipher the complexities of lncRNA expression in breast.

This study presents the first evidence for the presence of a link between the lncRNA expression and the reproductive factors or obesity in the breast tissue of healthy women. In conclusion, the expression levels of GAS5 and LSINCT5 lncRNAs were related to the BMI subgroups, with both being downregulated in overweight-obese individuals. Furthermore, GAS5 was upregulated in women with late menarche.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

ORCID

Pantea Izadi \textsuperscript{12} http://orcid.org/0000-0002-5698-071X
Milad Bastami \textsuperscript{13} http://orcid.org/0000-0002-7686-4505

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