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ORIGINAL RESEARCH

Assessment of functional variants and expression of long noncoding RNAs in vitamin D receptor signaling in breast cancer

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Purpose: Vitamin D receptor (VDR) signaling pathway is implicated in the pathogenesis of breast cancer.

Patients and methods: We selected VDR-associated long noncoding RNAs (lncRNAs) through an in silico analysis of available microarray and RNA-sequencing data and assessed their expression in 75 breast tumor samples and their adjacent noncancerous tissues (ANCTs). We also genotyped two functional polymorphisms within *VDR* gene in all patients.

Results: *VDR*, *MALAT1*, and *LINC00511* were significantly upregulated in tumoral tissues compared with ANCTs (fold change [FC] =1.85, *P*=0.03; *FC* =1.54, *P*=0.04; and *FC* =4.75, *P*=0.000, respectively). In patients younger than 55 years, significant associations were found between expression levels of both *SNHG16* and *LINC00511* genes and nuclear grade (*P*=0.03), expression of *LINC00346* and tubule formation (*P*=0.01), expression of both *SNHG16* and *SNHG6* genes and family history of cancer (*P*=0.01 and 0.03, respectively), as well as expression of *VDR* and progesterone receptor status (*P*=0.03). We detected significant correlations between expression levels of *VDR* and *SNHG16* in both tumoral tissues and ANCTs. The TT genotype of FokI polymorphism was associated with the higher expression levels of *VDR*. FokI variants were associated with expression levels of both *MALAT1* and *SNHG16* in ANCTs (*P*=0.01 and 0.03, respectively). CdxII variants were associated with expression levels of *SNHG16* in ANCTs. A significant correlation was found between FC values of *SNHG16* expression and vitamin D levels.

Conclusion: The present study provides further evidence for the contribution of VDR signaling and the related lncRNAs in the pathogenesis of breast cancer and introduces some novel lncRNAs as putative molecules in the interactive functional network of VDR signaling in breast cancer.

Keywords: breast cancer, MALAT1, SNHG16, SNHG6, LINC00346, LINC00511, VDR

Introduction

Breast cancer is a prevalent cancer among women with a huge disease burden, which has been attributed to the lack of suitable biomarkers for the early detection of disease.[1] Despite several efforts to find diagnostic and prognostic biomarkers for breast cancer,[2–4] the clinical relevance of few biomarkers has been confirmed.[5] Vitamin D receptor (VDR) signaling pathway has been regarded as a putative biomarker and therapeutic target in breast cancer.[6] Vitamin D has potent antiproliferative, prodifferentiation, and proapoptotic functions in several tissues. Both autocrine VDR signaling and paracrine VDR signaling are important determinants of cell fate in diverse physiological and pathological contexts. In vitro and in vivo studies have provided evidence for the participation of VDR signaling in either chemoprevention or chemotherapy of breast cancer. In contrast, several genetic and epigenetic aberrations have been associated

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with resistance to VDR functions in cancers.[7] VDR binding to calcitriol leads to its translocation into the nucleus and the subsequent interactions with the vitamin D response element (VDRE), which results in the stimulation of the transcription of targeted genes among which are those involved in the carcinogenesis process.[2] VDR expression has been documented in several breast cancer cell lines[5,6] as well as clinical samples.[8,9] Moreover, an association has been found between low VDR expression in breast tumor tissues and aggressive tumor features such as large tumor size, hormone receptor negativity, and high Ki-67 expression.[9] Such studies have highlighted the role of VDR signaling in breast cancer. In contrast, VDREs have been detected in noncoding regions of the genome including those encompass the long noncoding RNA (lncRNA) genes.[10] These mRNA-like transcripts are involved in several carcinogenesis-associated cellular processes[11–13] and are abnormally expressed in breast tumors.[4,14,15] Besides, several lncRNAs have been shown to be regulated by VDR signaling in the context of skin cancer.[10] Taken together, we hypothesized that VDR expression might be associated with expression levels of certain lncRNAs and clinicopathological features of breast tumors. Consequently, we selected a number of VDR-associated lncRNAs through an in silico analysis of available microarray and RNA-sequencing data and assessed their expression in breast tumor samples compared with the adjacent noncancerous tissues (ANCTs). We also evaluated the associations between expression levels of these genes and tumor features such as stage and grade. In addition, we have genotyped two functional polymorphisms within *VDR* gene (FokI [rs2228570] and CdxII [rs11568820]) in all patients to evaluate their effects on the expression of *VDR* in breast samples.

Patients and methods

Patients

This study was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1396.804). All methods were performed in accordance with the relevant guidelines and regulations. Informed written consent was obtained from all patients. Seventy-five patients with definite diagnosis of invasive ductal carcinoma of breast who were referred to Sina and Farmanieh hospitals entered the study. Tumoral and ANCTs were excised during surgery and transferred in liquid nitrogen to the genetic laboratory for further assessments. Clinicopathological data of patients were collected through questionnaires and the assessment of medical records.

In silico analysis

We used the following online tools for the identification of lncRNAs, which interact with VDR and have functional significance in breast cancer: 1) LncRNA2Target tool (http://www.lncrna2target.org) for the detection of lncRNAs that function the upstream of VDR, 2) cBioPortal for Cancer Genomics (http://cbioportal.org), and 3) the Catalog of Somatic Mutations in Cancer (COSMIC) for the identification of lncRNAs that are targets of genomic alteration in breast cancer tissues.

SNPs genotyping

Genomic DNA was extracted from blood samples of patients using the standard salting out method as previously described.[16] The FokI (rs2228570) and CdxII (rs11568820) polymorphisms within *VDR* gene were genotyped using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) and tetra-primer amplification refractory mutation system–PCR (ARMS–PCR) techniques, respectively. Table 1 shows the nucleotide sequences of the primers used for SNPs' genotyping. The PCR program included an initial denaturation at 95°C for 5 minutes followed by 30 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds and a final extension step at 72°C for 5 minutes. For FokI (rs2228570) genotyping, the amplified PCR products were digested with FokI

Table 1 The nucleotide sequences of primers used for SNPs genotyping

Locus	Sequence 5′→3′	PCR product length	Digestion products
FokI	Forward: GGTGGGTGGCACCAAGGAT Reverse: CTCCTGTGGCTGTGAGCG	365 bp	CC:336, 29 bp CT: 336, 273, 63, 29 bp TT: 273, 63, 29 bp
CdxII	G-forward: AGGATAGAGAAAATAATAGAAAACATT G-reverse: AACCCATAATAAGAAATAAGTTTTTAC A-forward: TCCTGAGTAAACTAGGTCACAA A-reverse: ACGTTAAGTTCAGAAAGATTAATTC	Internal control: 297 bp A allele: 235 bp G allele: 110 bp	

Abbreviation: PCR, polymerase chain reaction.

restriction endonuclease enzyme (Thermo Fisher Scientific, Waltham, MA, USA) for 15 minutes at 37°C followed by inactivation step at 65°C for 5 minutes.

RNA extraction and quantitative real-time PCR

Expressions of *MALAT1* (ENSG00000251562), *SNHG16* (ENSG00000163597), *SNHG6* (ENSG00000245910), *LINC00346* (ENSG00000255874), *LINC00511* (ENSG00000227036), and *VDR* (ENSG00000111424) were assessed in tumoral tissues and ANCTs using the quantitative real-time PCR technique. In brief, total RNA was extracted from all samples using the TRIzol™ Reagent (Thermo Fisher Scientific). RNA samples were treated by DNase (Thermo Fisher Scientific) before cDNA synthesis. Next, cDNA was synthesized by using the RevertAid First Strand cDNA Synthesis Kit (TaKaRa, Otsu, Japan). The relative expression of *VDR* and mentioned lncRNAs was measured using the SYBR® Premix Ex Taq™ (TaKaRa) and application of *B2M* expression levels as normalizer. The nucleotide sequences of primers used for expression analysis are shown in Table 2. All experiments were performed in duplicate in the rotor gene 6000 corbett Real-Time PCR System.

Statistical analysis

Data were presented as mean ± SD. Student's paired and unpaired *t*-tests were used for the analysis of differences in gene expression between paired and unpaired samples. The association between clinicopathological data and transcript levels of each gene was assessed using the chi-square test.

Table 2 The nucleotide sequences of primers used for expression analysis

Gene	Sequence 5′→3′	PCR product (bp)
B2M	F: AGATGAGTATGCCTGCCGTG R: GCGGCATCTTCAAACCTCCA	105
VDR	F: GCCTTTGGGTCTGAAGTGTCT R: CCATTGCCTCCATCCCTGAA	94
MALAT1	F: GACGGAGGTTGAGATGAAGC R: ATTCGGGGCTCTGTAGTCCT	84
SNHG16	F: GTCAGCCTCAGTTTCCAAAGC R: TAAAGACATGGCACTTTGGGTC	104
SNHG6	F: AGGGAGGAAGAAGCGCGAA R: TCGCAGAGCCCAGCTACG	85
LINC00511	F: TCCCACCAGGAAGTTTAGCAG R: GCCTCTCAAGAGGTGGTCC	87
LINC00346	F: TGCCCTGGACATTCATGGAC R: CTGGACAAGCCCACTCTAGC	150

Abbreviation: PCR, polymerase chain reaction.

The expression fold change (FC) was measured using the $2^{-\Delta\Delta Ct}$ method. The pairwise correlation between vitamin D levels and expression of genes or between relative transcripts levels of genes was calculated using the regression model. For all statistical tests, the level of significance was set at $P<0.05$. We also depicted the receiver operating characteristic (ROC) curve to assess the suitability of gene expression levels for classifying disease status. In order to appraise the probability cutoff of gene expression, the Youden index (*J*) was applied to maximize the difference between sensitivity (true-positive rate) and 1- specificity (false-positive rate). The precision of each marker for the diagnosis of breast cancer was scored according to the area under curve (AUC) values using the following system: 0.90–1= excellent (A), 0.80–0.90= good (B), 0.70–0.80= fair (C), 0.60–0.70= poor (D), and 0.50–0.60= fail (F).

Results

General demographic data of patients

General demographic data of patients are shown in Table 3.

Genotyping

Genotyping of CdxII and FokI polymorphisms in breast cancer patients showed AA and CC genotypes as the most prevalent ones among patients, respectively. Table 4 shows the frequency of CdxII and FokI genotypes in breast cancer patients.

Relative expression of *VDR* and lncRNAs in tumoral tissues vs ANCTs

VDR, *MALAT1*, and *LINC00511* were significantly upregulated in tumoral tissues compared with ANCTs (FC =1.85, *P*=0.03; FC =1.54, *P*=0.04; and FC =4.75, *P*=0.000, respectively). For *MALAT1*, such upregulation was more prominent in patients elder than 55 years (FC =2.49, *P*=0.002). The expression levels of other lncRNAs were not significantly different between tumoral tissues and ANCTs.

Association between relative expression of genes and patients' clinicopathological data

No significant association was found between expression levels of genes and clinicopathological data of patients except higher expression levels of *SNHG16* in patients with positive family history of cancer (any cancer rather than breast cancer) (*P*=0.01). Then, we assessed associations between these clinicopathological data and relative expression of genes in

Table 3 General demographic data of study participants

Variables	Values
Age (years), mean ± SD (range)	51.53±12.83 (29–83)
Menarche age, mean ± SD (range)	13.1±1.56 (10–18)
Menopause age, mean ± SD (range)	44.62±14.89 (38–60)
First pregnancy age, mean ± SD (range)	18.97±8.26 (14–34)
Breast feeding duration (months), mean ± SD (range)	41.48±40.47 (3–240)
Serum level of 25-hydroxyvitamin D, mean ± SD (range)	22.85±13.98 (6.3–55.8)
Positive family history for cancer (%)	18.7
Cancer stage (%)	
I	25.3
II	31
III	33.8
IV	9.9
Overall grade (%)	
I	16.7
II	53
III	30.3
Nuclear grade (%)	
I	10.3
II	58.8
III	30.9
Tubule formation (%)	
I	6.5
II	33.9
III	59.6
Mitotic rate (%)	
I	40.9
II	42.9
III	16.2
Tumor size (%)	
<2 cm	26.1
≥2 to <5 cm	71
≥5 cm	2.9
ER (%)	
Positive	84.1
Negative	15.9
PR (%)	
Positive	77.9
Negative	22.1
Her2/neu expression (%)	
Positive	23.2
Negative	76.8
Ki67 expression (%)	
Positive	100
Negative	0

patients younger than 55 years. In this subgroup of patients, significant associations were found between *SNHG16* expression and nuclear grade (*P*=0.03), *LINC00511* expression and nuclear grade (*P*=0.03), and *LINC00346* expression and tubule formation (*P*=0.01). Higher levels of both *SNHG16* and *LINC00511* were more frequently detected in tumors with nuclear grade 2. Moreover, higher expression of *LINC00511*

Table 4 Frequency of CdxII and FokI genotypes in breast cancer patients

CdxII genotypes	Frequency, n (%)	FokI genotypes	Frequency, n (%)
AA	29 (38.7)	CC	46 (61.4)
AG	22 (29.3)	CT	25 (33.3)
GG	24 (32)	TT	4 (5.3)

was more commonly seen in tumors with tubule formation score 2. Besides, higher expression levels of both *SNHG16* and *SNHG6* genes were associated with the positive family history of cancer (*P*=0.01 and 0.03, respectively). In addition, a significant positive association was found between the relative expression of *VDR* and PR status (*P*=0.03). Table 5 shows the associations between the relative expression of these genes and clinicopathological data in patients younger than 55 years.

Correlation between relative expressions of genes

We assessed correlations between expression levels of genes in both tumoral tissues and ANCTs. The most significant correlations were found between expression levels of *VDR* and *SNHG16* in both tumoral tissues and ANCTs (Table 6).

Association between polymorphisms' genotypes and *VDR* and lncRNAs; relative expressions

FokI polymorphism was associated with the expression of *VDR* in both tumoral tissues and ANCTs (*P*=0.01 and 0.000, respectively) (Table 7). The TT and CC genotypes of this polymorphism were associated with the higher and lower expression levels of *VDR* (Figure 1).

Moreover, FokI variants were associated with expression levels of both *MALAT1* and *SNHG16* in ANCTs (*P*=0.01 and 0.03, respectively) but not tumoral tissues. CdxII variants were associated with expression levels of *SNHG16* in ANCTs (Table 7). Subsequent association analysis of CdxII genotypes with *VDR* expression in ER-based subgroups revealed no association in either ER-positive subtypes or ER-negative subtypes (data not shown).

Then, we assessed the associations between *VDR* polymorphisms and relative expressions of genes in tumoral tissues compared with the corresponding ANCTs and found significant over-representation of CC genotype in patients with the downregulation of both *VDR* and *LINC00511* genes in tumoral tissues compared with ANCTs (Table 8). No

association was found between CdxII genotypes and relative expression of genes.

Assessment of correlations between serum levels of vitamin D and expression of genes

We assessed the correlations between vitamin D levels and expression of genes in tumoral tissues and ANCTs, as well as relative expression of genes in tumoral vs ANCTs (FC values). A significant correlation was found between FC values of *SNHG16* expression and vitamin D levels (P=0.03). Table 9 shows the results of correlation analysis.

We also compared the serum levels of vitamin D in certain subgroups of patients based on the relative expression of each gene in tumoral tissues vs ANCTs and found no significant difference in vitamin D level between these groups. Figure 2 shows serum vitamin D levels in *SNHG16* up-/downregulated and *VDR* up-/downregulated subjects.

ROC curve analysis

Based on ROC curve analysis results, the accuracy of *LINC00511* expression levels for breast cancer diagnosis is fair (Figure 3). Besides, *MALAT1* and *VDR* expression levels were the most specific and sensitive diagnostic markers for breast cancer among the assessed markers, respectively. Table 10 shows the details of ROC curve analysis.

Discussion

In the present study, we evaluated expression levels of *VDR* and five lncRNAs with putative role in VDR signaling pathway in breast tumor samples vs ANCTs. *VDR* was significantly upregulated in breast tumor tissues compared with ANCTs. Its expression levels were associated with PR status in younger patients but not with other clinicopathological data or vitamin D levels. The observed association between *VDR* expression and PR status in this subgroup of patients is contrary to the results of Friedrich et al[18] and Ditsch et al[19] studies. Al-Azhri et al have reported the absence or low expression of VDR in 42% of breast tumors, moderate expression in 32% of breast tumors, and strong expression in the other 25% of samples using the immunohistochemistry (IHC) method. They found inverse association between VDR expression and more aggressive breast cancer behavior but no association between its expression and patients' survival. Moreover, they detected no association between vitamin D levels and VDR expression.[9] The latter finding is in accordance with our result regarding the lack of association between VDR expression in breast tissues and vitamin D levels. The discordance between our results and the mentioned studies can be explained by the difference in expression analysis method (quantitative real-time PCR vs IHC) and the assessment of relative expression in tumoral tissues vs ANCTs in our study but not in their studies.

We also demonstrated a significant overexpression of *MALAT1* in tumoral tissues compared with ANCTs. Miao et al have demonstrated *MALAT1* overexpression in 85.9% (67/78) of breast cancer tissues compared with ANCTs. They also have shown a significant association between *MALAT1* overexpression and lymph metastasis.[20] Moreover, Huang et al have reported *MALAT1* overexpression in ER-positive breast cancer samples compared with ANCTs. In addition, they have shown associations between its transcript levels and positive ER/PR status.[21] However, in the present study, we did not find any association between *MALAT1* expression levels and patients' clinicopathological data. Noticeably, *MALAT1* upregulation was more prominent in elderly patients. Such data are in line with the previously reported upregulation of *MALAT1* in chronic myelomonocytic leukemia (CMML) and its situation among the highest differentially expressed transcripts in CMML monocytes ($CD14^+$)[22] considering the facts that CMML is exceptionally rare in people younger than 50 years and its incidence sharply increases in people elder than 70 years.[23] Although the exact mechanism for age-related upregulation of *MALAT1* is not clarified, the previously reported higher frequency of somatic mutations in elderly breast cancer patients[24] might be implicated in this phenomenon.

In addition, we detected a significant upregulation of *LINC00511* in tumoral tissues compared with ANCTs. Besides, its expression was associated with nuclear grade in patients younger than 55 years. *LINC00511* has been found to be a highly scored differentially expressed transcript in ER-negative MDA-MB-231 cells compared with the ER-positive T-47D and MCF-7 cells. Its expression has been upregulated in retinoid-related orphan receptor (ROR) γ-depleted MCF-7 and T-47D as well as aggressive basal-like breast cancer subtype.[25] In contrast, RORγ function has been shown to be regulated by CYP11A1-derived vitamin D metabolites.[26] Consequently, *LINC00511* might provide the functional link between VDR signaling pathway and RORγ expression in breast cancer. However, such hypothesis should be evaluated in future studies. Moreover, next-generation sequencing of HER-2-enriched subtype breast cancer has shown *LINC00511* as one of the core genes in the dysregulated pathways in this type of breast cancer including MAPK signaling pathway, PI3K–Akt signaling pathway, metabolic

Table 5 The associations between relative expression of these genes and clinicopathological data in patients younger than 55 years

Clinicopathological Features	*VDR* up-regulation, n (%)	*VDR* down-regulation, n (%)	*P*-value	*MALAT1* up-regulation, n (%)	*MALAT1* down-regulation, n (%)	*P*-value	*SNHG16* up-regulation, n (%)	*SNHG16* down-regulation, n (%)
Stage			0.94			0.73		
1	5 (50)	5 (50)		5 (50)	5 (50)		4 (40)	6 (60)
2	8 (61.5)	5 (38.5)		5 (33.3)	10 (66.7)		7 (46.7)	8 (53.3)
3	9 (64.3)	5 (35.7)		8 (53.3)	7 (46.7)		9 (64.3)	5 (35.7)
4	3 (60)	2 (40)		2 (40)	3 (60)		2 (40)	3 (60)
Histological grade			0.19			0.1		
1	5 (71.4)	2 (28.6)		6 (75)	2 (25)		5 (62.5)	3 (37.5)
2	14 (70)	6 (30)		9 (40.9)	13 (59.1)		12 (57.1)	9 (42.9)
3	4 (36.4)	7 (63.6)		3 (27.3)	8 (72.7)		3 (27.3)	8 (72.7)
Nuclear grade			0.31			0.14		
1	2 (40)	3 (60)		5 (83.3)	1 (16.7)		3 (50)	3 (50)
2	17 (70.8)	7 (29.2)		12 (46.2)	14 (53.8)		17 (68)	8 (32.2)
3	5 (50)	5 (50)		3 (30)	7 (70)		2 (20)	8 (80)
Tubule formation			0.09			0.18		
1	1 (100)	0 (0)		2 (100)	0 (0)		1 (50)	1 (50)
2	10 (83.3)	2 (16.7)		7 (50)	7 (50)		9 (69.2)	4 (30.8)
3	10 (47.6)	11 (52.4)		7 (33.3)	14 (66.7)		9 (42.9)	12 (57.1)
Mitotic rate			0.24			0.16		
1	9 (81.8)	2 (18.2)		8 (66.7)	4 (33.3)		8 (66.7)	4 (33.3)
2	8 (53.3)	7 (46.7)		5 (29.4)	12 (70.6)		8 (50)	8 (50)
3	4 (50)	4 (50)		3 (37.5)	5 (62.5)		3 (37.5)	5 (62.5)
Tumor size			0.21			1		
<2	5 (55.6)	4 (44.4)		5 (50)	5 (50)		6 (66.7)	3 (33.3)
2–5	19 (65.5)	10 (34.5)		14 (45.2)	17 (54.8)		15 (45.2)	16 (51.6)
>5	0 (0)	2 (100)		1 (50)	1 (50)		1 (50)	1 (50)
Family history			0.32			0.05		
+	5 (55.6)	4 (44.4)		7 (77.8)	2 (22.2)		8 (88.9)	1 (11.1)
−	19 (65.5)	10 (34.5)		14 (37.8)	23 (62.2)		14 (39.1)	22 (61.1)
ER status			0.24			1		
+	21 (63.6)	12 (36.4)		16 (45.7)	19 (54.3)		19 (55.9)	15 (44.1)
−	3 (37.5)	5 (62.5)		4 (44.4)	5 (55.6)		3 (33.3)	6 (66.7)
PR status			0.03			0.73		
+	22 (66.7)	11 (33.3)		16 (47.1)	18 (52.9)		19 (55.9)	15 (44.1)
−	2 (25)	6 (75)		4 (40)	6 (60)		3 (33.3)	6 (66.7)
Her2 status			0.27			0.17		
+	4 (40)	6 (60)		3 (27.3)	8 (72.7)		5 (45.5)	6 (54.5)
−	20 (64.5)	11 (35.5)		18 (52.9)	16 (47.1)		17 (51.5)	16 (48.5)

Notes: The REx of each gene in the tumoral tissue compared with the ANCT of the same patient was assessed. REx values >1 were described as "upregulation", while REx values ≤1 pointed to "downregulation". TNM staging was performed based on the AJCC staging guidelines (seventh edition).[17] Histological grade was calculated by the combination of scores obtained from nuclear grade, mitotic rate, and tubule formation. Nuclear grade scores were based on the appearance of the nucleus of the cancer cells, with one being the nearest to normal cells and three being the most deviation. Mitotic rate scores 1 and 3 were given to tumors with the slowest and the most rapid rate of mitosis. Tubule formation score represents the percentage of cancer cells that are in tubule formation. Tumor size refers to the greatest dimension of tumor in centimeters.

Abbreviations: AJCC, American Joint Committee on Cancer; ANCT, adjacent noncancerous tissue; REx, relative expression.

pathways, and those regulating cell cycle and actin cytoskeleton.[27] Taken together, our results are in line with the previous data regarding the crucial role of this lncRNA in breast cancer pathogenesis. Although the expression levels of other lncRNAs were not significantly different between tumoral tissues and ANCTs, significant associations were found between *LINC00346* expression and tubule formation as well as expression levels of both *SNHG16* and *SNHG6* genes and family history of cancer. Future studies are needed to elaborate the underlying mechanisms of such observations.

Next, we examined correlations between expression levels of genes in both tumoral tissues and ANCTs. *VDR* expression in tumoral tissues was correlated with the expression of all assessed lncRNAs except *LINC00511*. Notably, the most

***P*-value**	***SNHG6* up-regulation, n (%)**	***SNHG6* down-regulation, n (%)**	***P*-value**	***LINC00511* up-regulation, n (%)**	***LINC00511* down-regulation, n (%)**	***P*-value**	***LINC00346* up-regulation, n (%)**	***LINC00346* down-regulation, n (%)**	***P*-value**
0.67			0.14			0.78			0.31
	1 (14.3)	6 (85.7)		7 (70)	3 (30)		5 (50)	5 (50)	
	9 (69.2)	4 (30.8)		9 (69.2)	4 (30.8)		8 (66.7)	4 (33.3)	
	8 (53.3)	7 (46.7)		12 (80)	3 (20)		7 (50)	7 (50)	
	2 (40)	3 (60)		3 (60)	2 (40)		4 (100)	0 (0)	
0.24			0.62						0.55
	5 (71.4)	2 (28.6)		5 (62.5)	3 (37.5)		5 (71.4)	2 (28.6)	
	10 (47.6)	11 (52.4)		17 (81)	4 (19)		13 (65)	7 (35)	
	5 (45.5)	6 (54.5)		5 (50)	7 (54.5)		5 (45.5)	7 (54.5)	
0.03			0.79			0.03			0.27
	2 (50)	2 (50)		3 (50)	3 (50)		4 (80)	1 (20)	
	14 (56)	11 (44)		21 (84)	4 (16.2)		16 (66.7)	8 (33.3)	
	4 (40)	6 (60)		4 (44.4)	5 (55.6)		4 (40)	6 (60)	
0.32			0.42			1			0.01
	2 (100)	0 (0)		2 (100)	0 (0)		1 (50)	1 (50)	
	8 (61.5)	5 (38.5)		9 (69.2)	4 (30.8)		11 (97.7)	1 (8.3)	
	9 (45)	11 (55)		13 (65)	7 (35)		9 (42.9)	12 (57.1)	
0.43			0.18			0.1			1
	9 (75)	3 (25)		11 (91.7)	1 (8.3)		7 (58.3)	5 (41.7)	
	6 (40)	9 (60)		9 (56.3)	7 (43.8)		9 (60)	6 (40)	
	4 (50)	4 (50)		4 (57.1)	3 (42.9)		5 (62.5)	3 (37.5)	
0.72			1			0.71			1
	4 (50)	4 (50)		8 (80)	2 (20)		6 (66.7)	3 (33.3)	
	15 (53.6)	13 (46.4)		20 (69)	9 (31)		17 (63)	10 (37)	
	1 (50)	1 (50)		1 (50)	1 (50)		1 (50)	1 (50)	
0.01			0.03			1			0.44
	6 (85.7)	1 (14.3)		7 (77.8)	2 (22.2)		7 (77.8)	2 (22.2)	
	14 (41.2)	20 (58.8)		25 (71.4)	10 (28.6)		18 (56.3)	14 (43.8)	
0.28						1			1
	16 (51.6)	15 (48.4)	1	25 (71.4)	10 (28.6)		19 (61.3)	12 (38.7)	
	5 (55.6)	4 (44.4)		5 (71.4)	2 (28.6)		6 (66.7)	3 (33.3)	
0.28			0.85			0.69			1
	16 (5.3)	14 (46.7)		24 (72.7)	9 (27.3)		19 (61.3)	12 (38.7)	
	5 (50)	5 (50)		6 (66.7)	3 (33.3)		6 (66.7)	3 (33.3)	
1			0.29			0.42			0.52
	4 (36.4)	7 (63.6)		6 (60)	4 (40)		6 (54.4)	5 (45.5)	
	17 (5.6)	12 (41.4)		25 (75.8)	8 (24.2)		19 (65.5)	10 (34.5)	

significant correlations were found between expression levels of *VDR* and *SNHG16* in both tumoral tissues and ANCTs. Cai et al have reported frequent overexpression of *SNHG16* in breast tumor tissues compared with ANCTs and validated an oncogenic role for this lncRNA in breast cancer. Their in vitro studies indicated that *SNHG16* role in cell migration is exerted via miR-98.[28] In contrast, Ting et al[29] have reported the transcriptional induction of miR-98 by 1α,25-dihydroxy vitamin D3 in LNCaP cells. Taken together, these data suggest the presence of an interactive network between *SNHG16*, *VDR*, and miR-98 in the context of cancer. The observed correlation between *SNHG16* expression ration and serum vitamin D further supports this hypothesis. Assessment of correlations between transcript levels of these genes also revealed context-dependent correlations. For instance, the correlation between *LINC00346* and *SNHG16* was seen

only in ANCTs, while the correlation between *LINC00511* and *SNHG16* was only detected in tumoral tissues. Such observation might indicate the presence of context-dependent regulatory mechanisms for these lncRNAs, which need to be assessed in future studies.

Table 6 Coefficients of determination (R^2) values between expression levels of genes in both tumoral tissues and ANCTs

	LINC00346	*LINC00511*	*SNHG6*	*SNHG16*	*Malat1*
VDR					
Tumor	0.06*	0	0.05*	0.18*	0.07*
ANCT	0.08*	0.04	0.07	0.31*	0.11*
MALAT1					
Tumor	0.03	0.08*	0.01	0.01	
ANCT	0.07*	0	0.03	0	
SNHG16					
Tumor	0.04	0.06*	0.08*		
ANCT	0.11*	0.03	0.12*		
SNHG6					
Tumor	0.05	0.12*			
ANCT	0.05	0.07*			
LINC00511					
Tumor	0.05				
ANCT	0				

Note: *Correlation is significant at $P<0.05$ level.
Abbreviation: ANCTs, adjacent noncancerous tissues.

We also evaluated VDR expression in tumoral tissues and ANCTs in relation with FokI and CdxII genotypes. FokI polymorphism was associated with the expression of *VDR* in both tumoral tissues and ANCTs. The TT and CC genotypes of this polymorphism were associated with the higher and lower expression levels of *VDR*. In addition, we found significant over-representation of CC genotype in patients with the downregulation of *VDR* in tumoral tissues compared with ANCTs. FokI polymorphism is located in the promoter region 5′ of exon 2. Previous studies have indicated that the C>T change results in the production of a longer protein that is less operative as a transcriptional activator of VDR.[30] From our observations in breast cancer patients, it can be deduced that CC genotype can result in a more prominent downregulation of VDR in tumoral tissues compared with ANCTs. Moreover, FokI variants were associated with expression levels of both *MALAT1* and *SNHG16* in ANCTs, but not tumoral tissues, which provide further evidence for distinct regulation of these lncRNAs in tumoral

Table 7 Association between relative expression (delta CT mean ± SD) of genes and *VDR* polymorphisms

CdxII					**FokI**				
Alleles	**Expression in ANCTs**	***P*-value**	**Expression in tumoral tissues**	***P*-value**	**Alleles**	**Expression in ANCTs**	***P*-value**	**Expression in tumoral tissues**	***P*-value**
VDR		0.87		0.46			0.000		0.01
AA	11.01±3.31		9.25±2.57		CC	12.4±2.72		10.21±2.13	
AG	10.91±3.91		9.74±2.23		CT	8.41±2.05		9.29±2.92	
GG	10.53±2.53		10.15±2.89		TT	7.01±2.51		6.74±2.56	
MALAT1		0.63		0.61			0.01		0.25
AA	−2.6±1.67		−3.01±2.19		CC	−2.1±1.81		−3.37±2.47	
AG	−2.87±1.95		−3.68±2.92		CT	−3.34±1.49		−3.25±1.78	
GG	−2.6±1.75		−3.13±2.09		TT	−2.24±1.6		−1.3±3.69	
SNHG16		0.03		0.89			0.03		0.59
AA	4.61±1.46		4.25±2.61		CC	3.81±3.28		4.12±1.23	
AG	3.73±1.58		4.06±1.56		CT	3.69±1.51		4.5±3.05	
GG	3.62±1.27		4.34±1.68		TT	4.05±1.6		3.53±1.64	
SNHG6		0.05		0.35			0.44		0.01
AA	3.06±1.39		2.73±1.35		CC	3.07±1.52		2.85±1.27	
AG	3.45±1.56		3.46±2.45		CT	2.87±1.41		3.59±2.07	
GG	2.37±1.31		2.93±1.27		TT	2.12±0.85		1.17±2.28	
LINC00511		0.29		0.56			0.1		0.29
AA	16.24±5.63		12.49±4		CC	16.42±5.37		11.75±3.25	
AG	16.28±4.78		11.65±2.96		CT	14.65±5.93		12.96±3.66	
GG	13.99±6.2		12.68±2.82		TT	10.93±2.88		13.35±2.08	
LINC00346		0.31		0.83			0.74		0.44
AA	13.44±3.11		12.65±3.42		CC	13.75±3.3		11.86±3.85	
AG	14.51±3.95		12.08±3.21		CT	13.92±5.68		13.19±4.05	
GG	12.49±5.47		11.99±5.65		TT	13.55±2.12		12.78±8.45	

Abbreviation: ANCTs, adjacent noncancerous tissues.

and nontumoral tissues. CdxII variants were not associated with expression levels of VDR in any sets of tissues, but these variants were associated with expression levels of *SNHG16* in ANCTs. Pulito et al[31] have found a correlation between VDR mRNA, protein levels, and CdxII genotypes in the ER negative breast cancer cell lines in spite of the lack of such correlations in two ER-positive cell lines. They also reported associations between the AA genotype and low levels of VDR in the majority of breast cancer patients. When they categorized their patients by molecular subtypes, the association between CdxII polymorphism and VDR expression was detected only in Her2-positive and triple-negative subtypes.[31] However, we did not find any associations between CdxII polymorphisms and VDR expression in either ER-positive samples or ER-negative samples. Such discordance might be due to low number of ER negative samples (n=11) in our cohort of patients. The observed associations between *VDR* polymorphisms and the expression levels of certain genes as demonstrated in our study highlight the importance of genotyping of these variants before administration of novel VDR-targeted therapies in cancer patients.

The lncRNAs whose expressions have been assessed in the present study have several mRNA and miRNA targets themselves. For instance, previously published data have shown interaction of *MALAT1* with *ER*,[21] miR-129-5p[32],

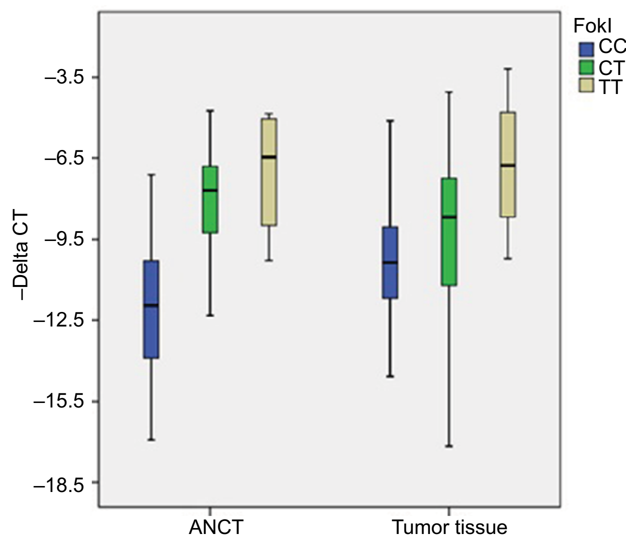


Figure 1 Relative expression of *VDR* (-delta CT values= CT_{B2M} - CT_{VDR}) in association with different genotypes of FokI polymorphism.
Abbreviations: ANCT, adjacent noncancerous tissue; CT, cycle threshold.

Table 8 Association between *VDR* polymorphisms and relative expressions of genes in tumoral tissues compared with the corresponding ANCTs

CdxII				**FokI**			
Alleles	**Upregulated, n (%)**	**Downregulated, n (%)**	***P*-value**	**Alleles**	**Upregulated, n (%)**	**Downregulated, n (%)**	***P*-value**
VDR			0.32				0.01
AA	20 (69)	9 (31)		CC	31 (70.5)	13 (29.5)	
AG	9 (47.4)	10 (52.6)		CT	8 (34.8)	15 (65.2)	
GG	13 (59.1)	9 (40.9)		TT	3 (75)	1 (25)	
MALAT1			0.8			0.07	0.07
AA	14 (48.3)	15 (51.7)		CC	29 (63)	17 (37)	
AG	12 (57.1)	9 (42.9)		CT	9 (36)	16 (64)	
GG	12 (52.2)	11 (47.8)		TT	2 (50)	2 (50)	
SNHG16			0.59				0.29
AA	16 (55.2)	13 (44.8)		CC	24 (52.2)	22 (47.8)	
AG	10 (47.6)	11 (52.4)		CT	9 (37.5)	15 (62)	
GG	9 (40.9)	13 (59.1)		TT	2 (75)	1 (25)	
SNHG6			0.55				0.16
AA	15 (55.6)	12 (44.4)		CC	23 (59)	16 (41)	
AG	10 (52.6)	9 (47.4)		CT	8 (33.3)	16 (66.7)	
GG	8 (40)	12 (60)		TT	2 (50)	2 (50)	
LINC00511			0.88				0.01
AA	21 (72.4)	8 (27.6)		CC	36 (83.7)	7 (16.3)	
AG	15 (75)	5 (25)		CT	15 (60)	10 (40)	
GG	15 (68.2)	7 (31.8)		TT	1 (25)	3 (75)	
LINC00346			0.71				0.63
AA	18 (64.3)	10 (35.7)		CC	27 (61.4)	17 (38.6)	
AG	11 (55)	9 (45)		CT	15 (65.2)	8 (34.8)	
GG	14 (66.7)	7 (33.3)		TT	1 (33.3)	2 (66.7)	

Abbreviation: ANCTs, adjacent noncancerous tissues.

DBC1,[33] miR-204/ZEB2 axis,[34] *CD133*,[35] miR-204,[36] *CCND1*,[37] *Sox-2*,[38] miR-1,[39] miR-124,[40] and *VEGFA*.[41] Moreover, *SNHG16* and *LINC00511* interact with miR-98/E2F5[28] and *SOX9*,[25] respectively. So, future studies are needed to

Table 9 The correlations between vitamin D levels and expression of genes were assessed in tumoral tissues and ANCTs separately

Genes	ANCT		Tumor tissue		Rex	
	R^2	*P*-value	R^2	*P*-value	R^2	*P*-value
VDR	0.067	0.31	0.068	0.31	0.008	0.73
MALAT1	0.018	0.6	0.007	0.74	0.001	0.9
SNHG16	0.118	0.17	0.011	0.68	0.263	0.03
SNHG6	0.028	0.554	0.001	0.89	0.009	0.73
LINC00511	0.004	0.82	0.001	0.92	0.025	0.55
LINC00346	0.078	0.29	0.001	0.92	0.008	0.73

Notes: Moreover, we calculated REx of each gene in tumoral tissue compared with the ANCT of the same patient and assessed the correlation between REx value of each gene and vitamin D level in each patient.
Abbreviations: ANCTs, adjacent noncancerous tissues; REx, relative expression.

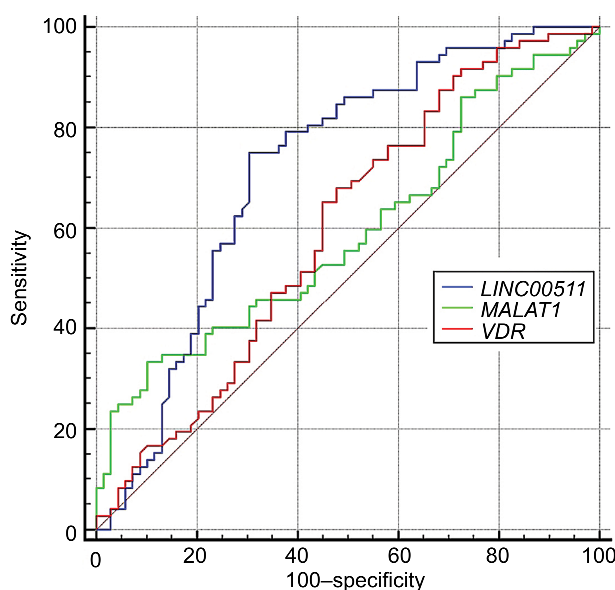
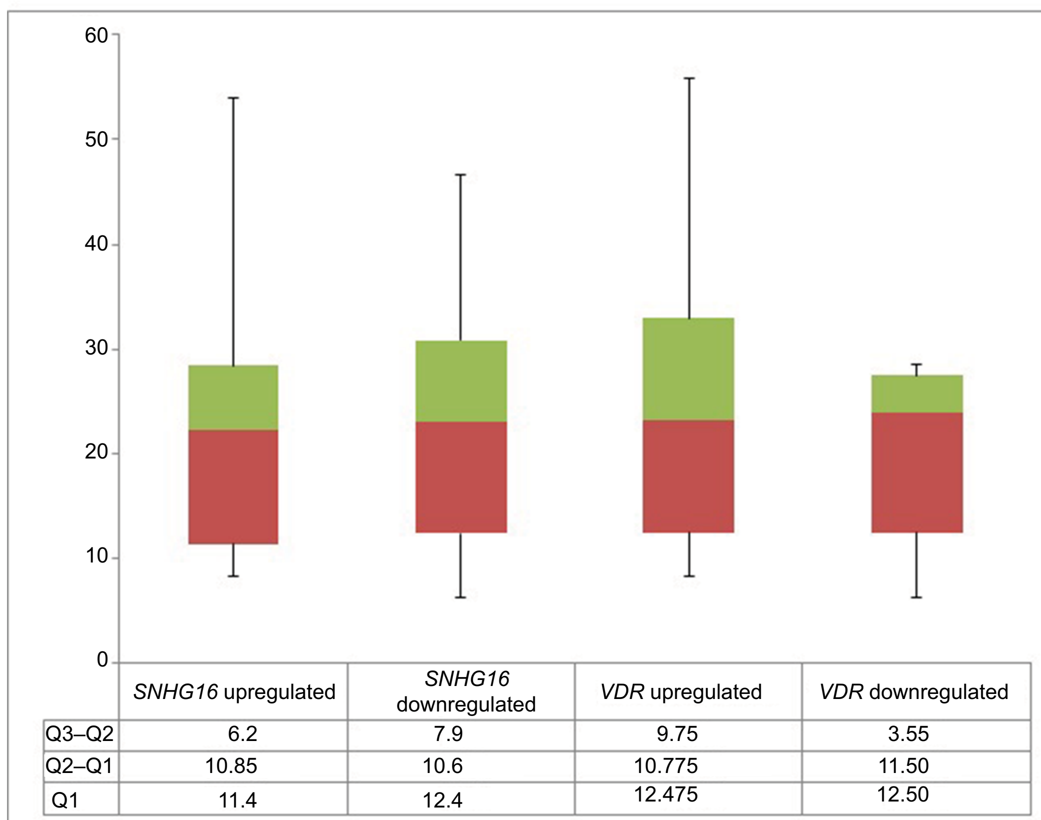


Figure 3 ROC curve for the assessment of *LINC00511*, *MALAT1*, and *VDR* expression levels as a diagnostic marker for breast cancer.
Abbreviation: ROC, receiver operating characteristic.



	SNHG16 upregulated	*SNHG16* downregulated	*VDR* upregulated	*VDR* downregulated
Q3–Q2	6.2	7.9	9.75	3.55
Q2–Q1	10.85	10.6	10.775	11.50
Q1	11.4	12.4	12.475	12.50

Figure 2 Serum vitamin D levels in *SNHG16* up-/downregulated and *VDR* up-/downregulated subjects.
Notes: The REx of each gene in the tumoral tissue compared with the ANCT of the same patient was assessed. REx values >1 were described as "up-regulation" while REx values ≤1 pointed to "downregulation".
Abbreviations: ANCT, adjacent noncancerous tissue; REx, relative expression.

Table 10 The results of ROC curve analysis

	Estimate criterion	AUC	J[a]	Sensitivity	Specificity	P-value[b]
LINC00511	>13.14	0.716	0.43	75	68.06	<0.0001
MALAT1	≤-4.33	0.596	0.226	32	90.67	0.03
VDR	≤12	0.597	0.19	87.84	31.94	0.04

Notes: Estimate criterion: optimal cutoff point for gene expression. [a]Youden index. [b]Significance level *P* (area =0.5).
Abbreviations: AUC, area under curve; ROC, receiver operating characteristic.

assess the dysregulation of all of these targets in the context of breast cancer in an integrative manner.

Finally, we assessed the accuracy of the three dysregulated genes (*LINC00511*, *MALAT1*, and *VDR*) for the diagnosis of breast cancer using ROC curve analysis but did not find high performance for any of them. However, *LINC00511* expression levels had fair accuracy for such purpose. Besides, *MALAT1* and *VDR* expression levels were the most specific and sensitive diagnostic markers for breast cancer among the assessed markers, respectively. Taken together, none of these genes have the ideal features to be used as an individual biomarker. Future studies are needed to assess the accuracy of a panel of these genes in the diagnosis of breast cancer.

Conclusion

The present study provides further support for the contribution of VDR signaling and the related lncRNAs in the pathogenesis of breast cancer and introduces some novel lncRNAs as putative molecules in the interactive functional network of VDR signaling in breast cancer.

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Author contributions

VKO conducted the experiments and the data analyses. LG contributed to the evaluation of patients. MDO participated in the study design and coordination. SG-F designed and supervised the study and wrote the manuscript. All authors read the manuscript drafts, contributed to the edits, and approved the final manuscript. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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