

Original Article

Peripheral Blood Leukocyte ANRIL Expression as a Potential Non-Invasive Biomarker for Breast Cancer: Associations with Oxidative Stress, Inflammation, and TCGA Validation

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Abstract

Background: This study evaluated long non-coding RNA ANRIL expression in peripheral blood leukocytes of patients with breast cancer and its diagnostic value in relation to oxidative stress and inflammation.**Methods:** This case-control study included 29 newly diagnosed patients with breast cancer and 30 healthy controls. ANRIL expression was quantified by quantitative real-time polymerase chain reaction. Malondialdehyde, total antioxidant capacity, superoxide dismutase, and plasma interleukin-6 were measured using commercial assays. Diagnostic performance was assessed by receiver operating characteristic analysis. The Cancer Genome Atlas Breast Invasive Carcinoma data were analyzed for differential expression, co-expression, oxidative stress-related gene correlations, and functional enrichment.**Results:** ANRIL expression showed a decreasing trend in patients with breast cancer versus controls ($P = 0.05$). Patients had higher malondialdehyde and interleukin-6 levels and lower total antioxidant capacity and superoxide dismutase activity (all $P < 0.001$). No significant correlations were observed between ANRIL and oxidative stress markers or plasma interleukin-6. Receiver operating characteristic analysis showed good discrimination by ANRIL (area under the curve = 0.864). In The Cancer Genome Atlas Breast Invasive Carcinoma data, ANRIL and interleukin-6 showed a weak but significant inverse correlation ($r = -0.114$, $P < 0.001$). Functional enrichment indicated that ANRIL-associated genes were mainly involved in cell-cycle regulation and mitosis.**Conclusions:** ANRIL showed a decreasing trend and good discriminatory performance in breast cancer. Although no direct association was observed with the assessed oxidative stress or inflammatory markers, transcriptomic analysis linked ANRIL to cell-cycle- and mitosis-related pathways. Larger multicenter and mechanistic studies are needed to validate these findings.

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1. INTRODUCTION

Breast cancer is one of the most common causes of cancer-related mortality among women, particularly in industrialized countries (1). Early detection through screening programs plays a critical role in reducing mortality and improving patient prognosis. Although several imaging modalities, including mammography, ultrasonography, and magnetic resonance imaging, are routinely used for breast cancer screening, these approaches often lack sufficient sensitivity and specificity, leading to false-positive or false-negative results. Therefore, the development of reliable, accurate, non-invasive, and cost-effective diagnostic biomarkers for the detection of breast tissue abnormalities remains a major clinical need (2, 3).

Recent advances in genomic and transcriptomic analyses have revealed that although more than 90% of the human genome is actively transcribed (4), protein-coding genes constitute less than 2% of the total transcribed genome, whereas the majority of transcripts correspond to non-coding RNAs (ncRNAs) (5). Numerous studies have demonstrated that dysregulation of ncRNAs is closely associated with various diseases, including different types of cancer such as lung, breast, colorectal, and bladder cancers (5, 6). Long non-coding RNAs (lncRNAs) are a class of ncRNAs characterized by two major features: they lack significant protein-coding potential and are generally longer than 200 nucleotides (7). lncRNAs share several characteristics with messenger RNAs (mRNAs), including transcription by DNA-dependent RNA polymerases, the presence of a 5' cap and poly-A tail, and alternative splicing events. Moreover, their biological functions are largely mediated through their complex tertiary structures (8).

Accumulating evidence indicates that lncRNAs play essential roles in various cellular processes, including cell proliferation, apoptosis, tumor progression, and metastasis (9, 10). They regulate the expression of associated genes at both transcriptional and post-transcriptional levels (11). Furthermore, due to their stability in biological fluids such as blood and urine, as well as their tissue-specific expression patterns, lncRNAs have emerged as promising diagnostic, prognostic, and therapeutic biomarkers in cancer research (12-14). Altered expression of lncRNAs may contribute to breast cancer development through oncogenic or tumor-suppressive mechanisms (15).

Among cancer-associated lncRNAs, the antisense non-coding RNA in the INK4 locus (ANRIL), located at chromosome 9p21, has attracted considerable attention because of its crucial role in tumor progression. ANRIL regulates gene expression through recruitment of the

polycomb repressive complex 2 (PRC2) and modulation of microRNA activity, thereby influencing multiple signaling pathways involved in carcinogenesis (16). Overexpression of ANRIL has been reported in various malignancies and is associated with enhanced cell proliferation, cell cycle progression, inhibition of apoptosis, and suppression of cellular senescence. In addition, elevated ANRIL expression has been correlated with advanced tumor stage, lymph node metastasis, higher histological grade, and poor overall survival in cancer patients. These findings suggest that ANRIL may serve as a novel biomarker for cancer diagnosis and prognosis. Moreover, inhibition of ANRIL expression has been shown to suppress tumor cell growth and migration through modulation of important signaling pathways such as PI3K/Akt (16).

In addition to its role in cancer, ANRIL has been identified as a key regulatory molecule in chronic diseases associated with oxidative stress and inflammation, including stroke and diabetes. Experimental studies have shown that increased ANRIL expression activates the NF- κ B signaling pathway, leading to enhanced inflammatory responses and apoptosis, whereas its suppression reduces inflammation and improves cellular function (17). In diabetic nephropathy, ANRIL upregulation under hyperglycemic conditions promotes oxidative stress and inflammatory responses in renal podocytes, while its silencing is associated with increased expression of protective genes such as MME (18). Similarly, in cancer, elevated ANRIL expression contributes to tumor progression and resistance to apoptosis through inflammatory and oxidative stress-related mechanisms (19).

Considering the unique properties of lncRNAs, including their tissue specificity, stability in biological fluids, and detectability in peripheral blood, these molecules may represent valuable non-invasive biomarkers for cancer diagnosis and monitoring. Therefore, the present study was designed to investigate the diagnostic potential of ANRIL expression in peripheral blood leukocytes of breast cancer patients and to evaluate its association with oxidative stress markers, particularly Total Antioxidant Capacity (TAC), and inflammatory indices. To the best of our knowledge, no previous study has evaluated ANRIL expression in peripheral blood leukocytes of patients with breast cancer.

2. MATERIALS AND METHODS

2.1. Data Acquisition and Preprocessing (TCGA-BRCA)

Gene expression data and clinical information for invasive breast carcinoma (TCGA-BRCA) were retrieved from The Cancer Genome Atlas (TCGA) using the TCGAbiolinks

package in R (v2.30.0). RNA sequencing data processed via the STAR-Counts pipeline included 1,098 tumor samples and 114 adjacent normal tissues. Raw unstranded read counts were extracted using the SummarizedExperiment object. Genes with low expression (mean count < 10 across samples) were excluded, except for ANRIL and IL6. Variance-stabilizing transformation (VST) was applied using DESeq2 (v1.42), and counts per million (CPM) were calculated using edgeR. Clinical variables, including tumor status, PAM50 subtype, survival outcomes, age at diagnosis, and disease stage, were obtained from the colData slot.

2.2. Differential Expression and Diagnostic Analysis

Expression levels of ANRIL (ENSG00000240498) and IL6 (ENSG00000136244) were compared between tumor and normal tissues using VST-transformed data. Diagnostic performance was evaluated using receiver operating characteristic (ROC) curve analysis with the pROC package, and the area under the curve (AUC) with 95% confidence intervals was calculated.

2.3. Survival and Prognostic Analysis

Overall survival (OS) was defined as the time from diagnosis to death or last follow-up. Kaplan-Meier survival curves were generated using the survival and survminer packages. Patients were stratified into high- and low-expression groups based on median gene expression values. Univariate and multivariate Cox proportional hazards models were used to estimate hazard ratios (HRs) and 95% confidence intervals. A combined risk score was constructed using a multivariate Cox model. Time-dependent ROC analysis was performed to evaluate prognostic performance at 3 years.

2.4. Co-expression Network Analysis

Pearson correlation coefficients between ANRIL and all protein-coding genes were calculated. Genes were ranked based on correlation strength, and the top 200 genes were selected. Significant correlations were defined as $|r| > 0.3$ with false discovery rate (FDR) < 0.05. Hierarchical clustering and heatmaps of the top 30 correlated genes were generated using the pheatmap package.

2.5. Weighted Gene Co-expression Network Analysis (WGCNA)

WGCNA was performed using the WGCNA package (v1.72) in R. The 10,000 most variable genes were selected for network construction. A soft-thresholding power ensuring scale-free topology ($R^2 > 0.85$) was applied.

Modules were identified using dynamic tree cutting with a minimum module size of 30 and a merge height of 0.25. Module-trait relationships were assessed using module eigengenes correlated with clinical features, including tumor status, age, and PAM50 subtype. Hub genes were identified based on intramodular connectivity, module membership (MM), and gene significance (GS).

2.6. Functional Enrichment Analysis

The top 100 genes most strongly correlated with ANRIL were subjected to functional enrichment analysis using the EnrichR platform. Enrichment was performed against KEGG_2021_Human, Reactome_2022, and GO_Biological_Process_2023 databases. Terms with adjusted p-value < 0.05 were considered statistically significant. Results were visualized using $-\log_{10}(\text{adjusted p-value})$ bar plots.

2.7. Correlation with Oxidative Stress-Related Genes

The association between ANRIL (CDKN2B-AS1; antisense non-coding RNA in the INK4 locus) expression and oxidative stress-related genes, including NOX4 (NADPH oxidase 4), GPX1 (glutathione peroxidase 1), GPX4 (glutathione peroxidase 4), SOD1 (superoxide dismutase 1), SOD2 (superoxide dismutase 2), HMOX1 (heme oxygenase 1), TXN (thioredoxin), TXNRD1 (thioredoxin reductase 1), PRDX1 (peroxiredoxin 1), and CAT (catalase), was evaluated using the GEPIA2 platform based on The Cancer Genome Atlas Breast Invasive Carcinoma (TCGA-BRCA) dataset. Pearson correlation analysis was performed using $\log_2(\text{TPM} + 1)$ -normalized expression values from tumor samples. Statistical significance was defined as $P < 0.05$.

2.8. Experimental Study Design

2.8.1. Study Population

A case-control study was conducted at Mazandaran University of Medical Sciences. Breast cancer patients were recruited from Shahid Rajaei Hospital, and healthy controls were selected from women attending routine clinical examinations. Patients had histopathologically confirmed primary breast cancer and had not received any prior chemotherapy, radiotherapy, or hormone therapy. Controls were frequency-matched by age (30–50 years). Individuals with autoimmune diseases, inflammatory conditions, infections, or other malignancies were excluded. Written informed consent was obtained, and the study was approved by the institutional ethics committee in accordance with the Declaration of Helsinki.

Table 1. Primer sequences used for qRT-PCR

Gene Name	Primer	Sequence
GAPDH	Forward	5'TGACTTCAACAGCGACACCCA-3'
	Reverse	5'CACCCTGTTGCTGTAGCCAAA-3'
ANRIL lncRNA	Forward	5'AGGCTGCTCCAGAACTGTAA-3'
	Reverse	5'TGGTTGCTCTGTGTCTGTTG-3'

Measurement of TAC, SOD Activity, and MDA Levels

Table 2. Comparison of oxidative stress and inflammatory parameters between control and cancer groups

Parameter	Control	Cancer	P value
MDA (nmol/mL)	9.06 ± 2.10	35.39 ± 4.32	0.0001
TAC (mmol/L)	4.17 ± 0.94	1.43 ± 0.53	0.0001
SOD (U/mL)	16.08 ± 5.60	10.20 ± 2.56	0.0001
IL-6 (pg/mL)	2.048 ± 0.09	5.390 ± 0.70	0.0002

2.8.2. Peripheral Blood Collection and RNA Processing

Peripheral blood samples (5 mL) were collected in EDTA tubes. Leukocytes were isolated using erythrocyte lysis buffer followed by centrifugation. Total RNA was extracted using TRIzol reagent (GeneAll, Korea) according to the manufacturer's protocol. RNA quantity and purity were assessed using a NanoDrop spectrophotometer. cDNA synthesis was performed using a reverse transcription kit (GeneAll, Korea).

2.8.3. Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed using SYBR Green Master Mix (Amplicon, Denmark) on an ABI 7500 system (Applied Biosystems, USA). ANRIL and GAPDH were used as target and housekeeping genes, respectively. All reactions were performed in triplicate. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Table 1).

2.8.4. Measurement of Oxidative Stress Markers

Total antioxidant capacity (TAC) was measured using a copper-reduction assay kit (Kiazist, Iran). Superoxide dismutase (SOD) activity was determined using the WST-1 method (Koshan Zist, Iran). Malondialdehyde (MDA) levels were assessed using the thiobarbituric acid reactive substances (TBARS) assay. All assays were performed according to manufacturer instructions.

2.8.5. Statistical Analysis

Statistical analyses were performed using R (v4.3.2) and SPSS. Continuous variables were expressed as mean ± standard deviation. Group comparisons were performed using independent t-test or Mann-Whitney U test. Pearson correlation analysis and ROC curve analysis were conducted to assess associations and diagnostic performance. A P-value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Demographic Characteristics of the Study Population

A total of 59 individuals, including 29 breast cancer patients and 30 healthy controls, were enrolled in the study. The mean BMI in the cancer and control groups was 30.39 ± 5.24 and 33.18 ± 7.23 kg/m², respectively, with no statistically significant difference between the groups ($P = 0.454$). Similarly, no significant difference was observed in age between breast cancer patients (48.75 ± 8.91 years) and healthy controls (45.43 ± 9.22 years) ($P = 0.751$).

3.2. Oxidative Stress and Inflammatory Biomarkers

Evaluation of oxidative stress parameters demonstrated a significant imbalance between oxidant and antioxidant systems in breast cancer patients compared with healthy controls. Plasma malondialdehyde (MDA) levels were markedly elevated in the cancer group (35.39 ± 4.32 nmol/mL) relative to controls (9.06 ± 2.10 nmol/mL; $P = 0.0001$), indicating enhanced lipid peroxidation and

oxidative stress. In contrast, total antioxidant capacity (TAC) and superoxide dismutase (SOD) activity were significantly reduced in breast cancer patients. TAC levels decreased from 4.17 ± 0.94 mmol/L in controls to 1.43 ± 0.53 mmol/L in the cancer group ($P = 0.0001$), while SOD activity declined from 16.08 ± 5.60 U/mL to 10.20 ± 2.56 U/mL, respectively ($P = 0.0001$). In addition, plasma interleukin-6 (IL-6) concentrations were significantly increased in breast cancer patients compared with healthy individuals (5.390 ± 0.70 vs. 2.048 ± 0.09 pg/mL, $P = 0.0002$), reflecting enhanced systemic inflammatory activity. However, no statistically significant correlation was observed between ANRIL expression levels and IL-6 concentrations in either the patient or control groups (Table2).

3.3. ANRIL Gene Expression Analysis

The ΔC_t values of ANRIL in breast cancer patients were higher compared to healthy controls (5.527 ± 1.833 vs. 4.773 ± 1.126 , mean $\pm$ SD). The difference between the two groups was statistically significant ($P = 0.05$) (Figure 1). The relative expression analysis using the $2^{-\Delta\Delta C_t}$ method showed that ANRIL expression in the cancer group was approximately 0.59-fold of that in the control group, indicating a reduction of about 41% in expression levels compared to healthy controls.

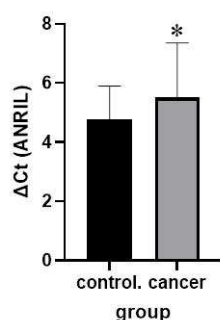


Figure 1. Relative ANRIL expression in control and cancer groups. ΔC_t values of ANRIL expression in control and cancer groups. Data are presented as mean $\pm$ SD. * $P < 0.05$.

3.4. Correlation Analysis Between ANRIL Expression and Oxidative Stress Markers

Pearson correlation analysis was conducted separately in the control and cancer groups to investigate the relationships between ANRIL expression and oxidative stress parameters (TAC, MDA, and SOD) (Table3).

In the control group, no statistically significant correlations were identified among the evaluated variables ($P > 0.05$).

However, a moderate positive correlation between ANRIL and TAC was observed ($r = 0.528$, $P = 0.063$), which approached statistical significance.

In the cancer group, a significant positive correlation was observed between TAC and SOD levels ($r = 0.717$, $P = 0.013$). No statistically significant associations were detected between ANRIL expression and oxidative stress markers, although ANRIL expression demonstrated a moderate positive correlation trend with MDA levels ($r = 0.366$, $P = 0.086$).

3.5. Correlation of ANRIL and IL6 Expression in the TCGA-BRCA Set

In 1,224 nonmetastatic TCGA-BRCA samples, including 1,111 primary tumor samples and 113 adjacent normal tissues, ANRIL and IL6 expression levels showed a weak but statistically significant negative correlation (Pearson's $r = -0.114$, $P = 6.53 \times 10^{-5}$; Spearman's $\rho = -0.113$, $P = 7.01 \times 10^{-5}$) (Figure 2A). However, the magnitude of the correlation was weak, indicating a limited linear association between ANRIL and IL6 expression.

Analysis according to PAM50 molecular subtypes revealed no statistically significant correlation between ANRIL and IL6 expression in any of the subtypes (Table 4). Among the evaluated subtypes, the strongest correlation was observed in the Basal-like subtype, although it was not statistically significant ($r \approx 0.07$, $P = 0.33$). In the Luminal A subtype, a weak negative correlation was observed, which was also not statistically significant ($r \approx -0.05$, $P = 0.22$) (Figure 2B–F).

3.6. Differential Expression and Diagnostic Performance

IL6 expression was significantly lower in tumor tissues than in adjacent normal tissues ($P < 0.05$), whereas ANRIL expression did not differ significantly between the two tissue types (Figure 3A and B). Receiver operating characteristic (ROC) curve analysis demonstrated good discriminatory performance for ANRIL (AUC = 0.864) and IL6 (AUC = 0.843) in distinguishing tumor from adjacent normal tissues (Figure 3C and D).

3.7. Prognostic value of ANRIL and IL6

Univariate Kaplan–Meier analysis showed that tumor ANRIL expression was significantly associated with overall survival (log-rank $P = 0.043$), although the observed effect was modest (Figure 4B). In contrast, plasma IL-6 levels were not significantly associated with overall survival (log-rank $P = 0.13$; Figure 4A). Combined stratification based on ANRIL and IL-6 levels showed a trend toward poorer overall

Table 3. Pearson correlation analysis of ANRIL, oxidative stress, and antioxidant parameters in control and cancer groups

Variable 1	Variable 2	Control Group r (P value)	Cancer Group r (P value)
ANRIL	TAC	0.528 (0.063)	-0.240 (0.259)
ANRIL	MDA	-0.080 (0.743)	0.366 (0.086)
ANRIL	SOD	-0.016 (0.955)	0.338 (0.201)
TAC	MDA	-0.273 (0.391)	0.051 (0.835)
TAC	SOD	-0.204 (0.548)	0.717 (0.013*)
MDA	SOD	-0.124 (0.687)	0.370 (0.263)

Statistically significant correlations are indicated by an asterisk (P < 0.05).

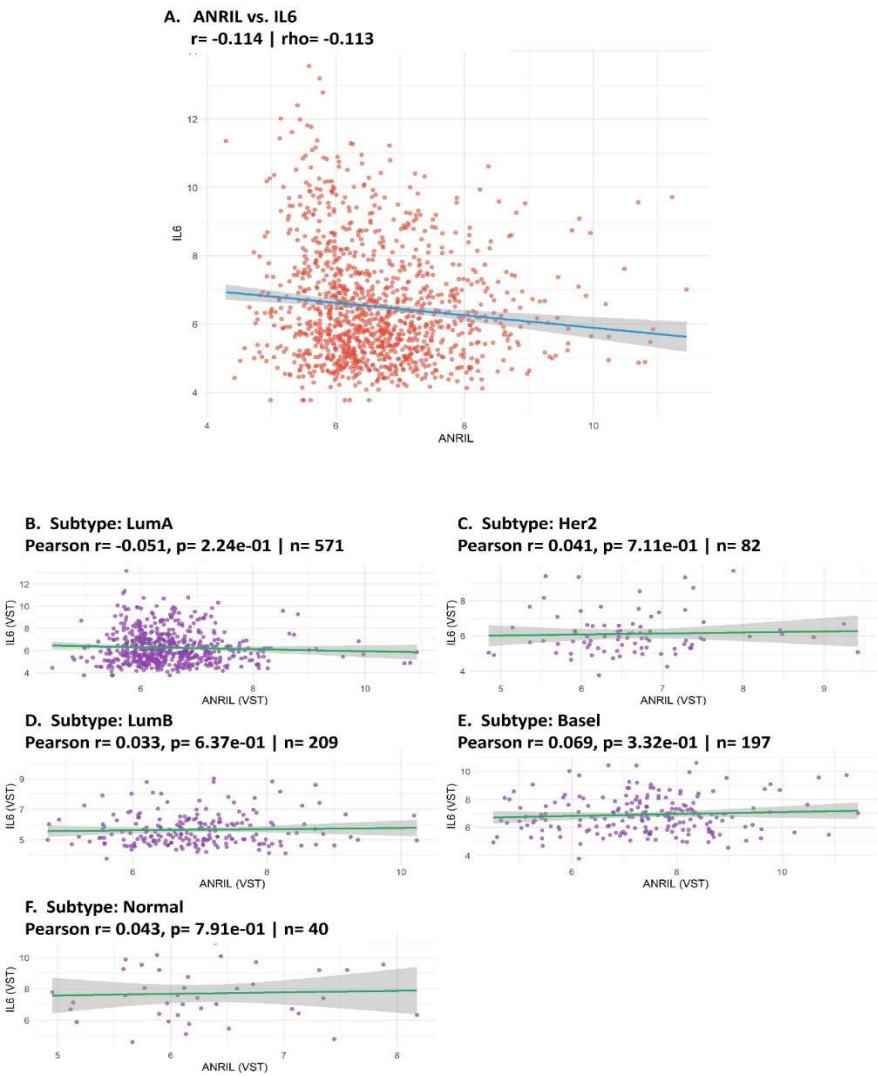


Figure 2. Correlation between ANRIL and IL6 expression. (A) Scatter plot in the entire cohort. (B–F) Subtype-specific scatter plots with linear regression lines and 95% confidence intervals.

Table 4. Pearson and Spearman correlation coefficients between ANRIL and IL6 expression stratified by PAM50 molecular subtypes in TCGA-BRCA cohort.

PAM50 Subtype	n	Pearson r	P-value	Spearman ρ	P-value
Basal-like	197	0.07	0.33	0.03	0.68
HER2-enriched	82	0.04	0.71	0.06	0.59
Luminal B	209	0.03	0.64	0.02	0.73
Luminal A	571	-0.05	0.22	-0.03	0.43
Normal-like	40	0.04	0.79	0.03	0.87

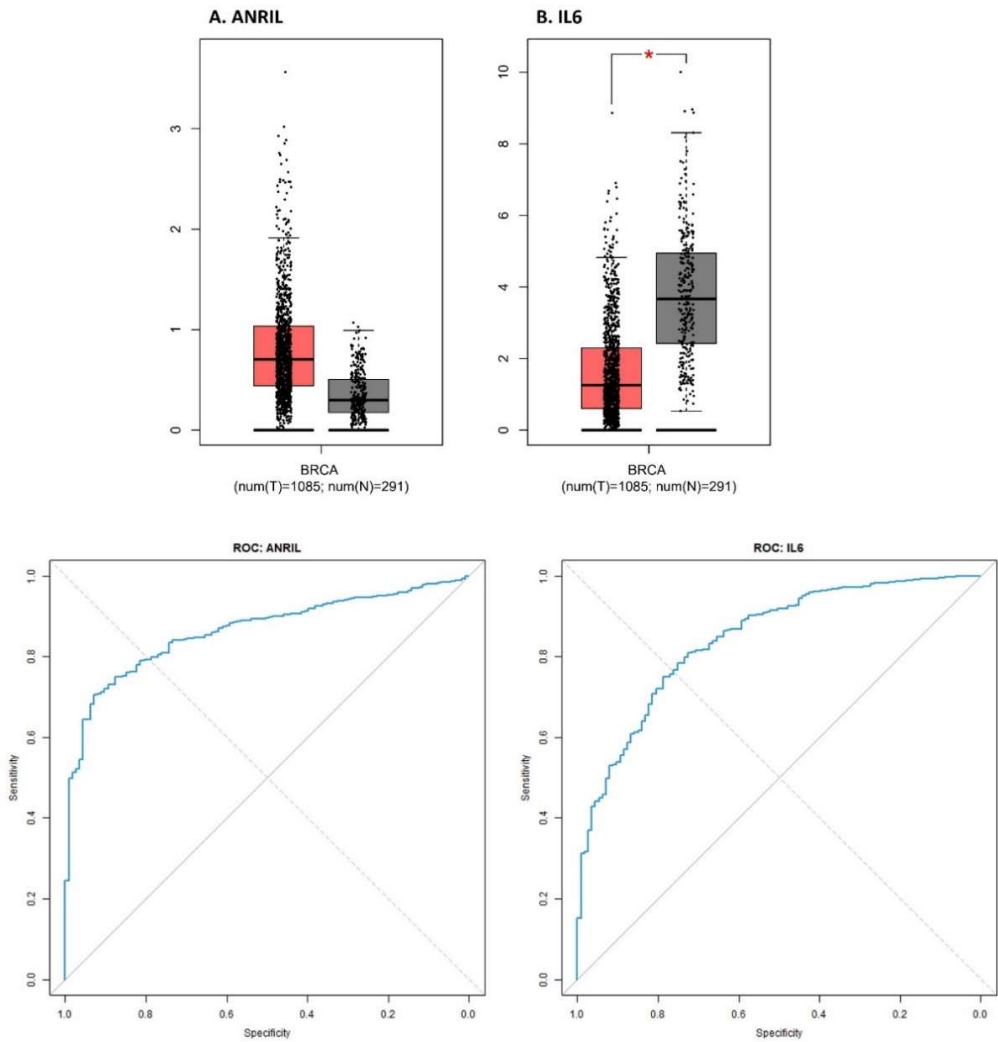


Figure 3. Differential analysis of (A) ANRIL and (B) IL6. Receiver operating characteristic (ROC) curves for discrimination of tumor versus normal breast tissue. (C) ANRIL (AUC = 0.864). (D) IL6 (AUC = 0.843).

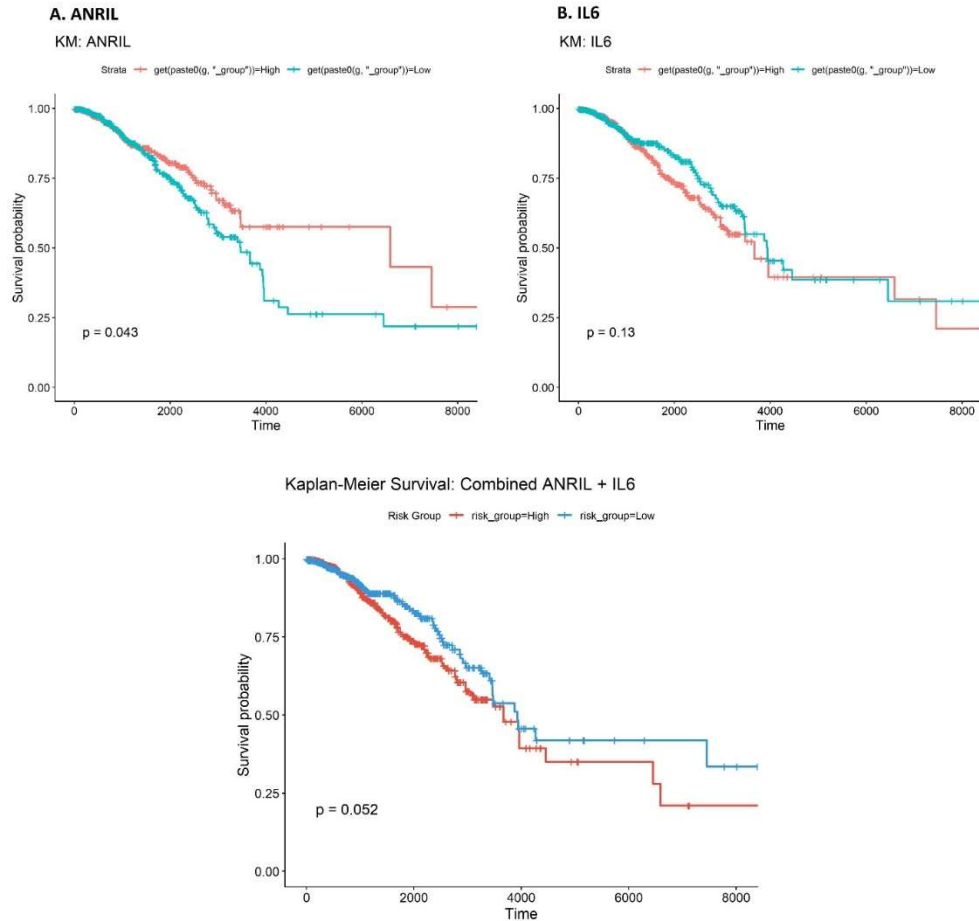


Figure 4. Prognostic value of IL-6 and ANRIL in breast cancer. (A) Kaplan-Meier overall survival curves stratified by tumor IL6 expression (high vs. low, cut-off: median; log-rank $P = 0.13$). (B) Kaplan-Meier curves stratified by tumor ANRIL expression (high vs. low, cut-off: median; log-rank $P = 0.043$). (C) Kaplan-Meier curves using a combined two-marker risk score (high-risk: high IL-6 + high ANRIL; low-risk: all others). The combined signature significantly stratified patients into distinct prognostic groups (log-rank $P = 0.052$).

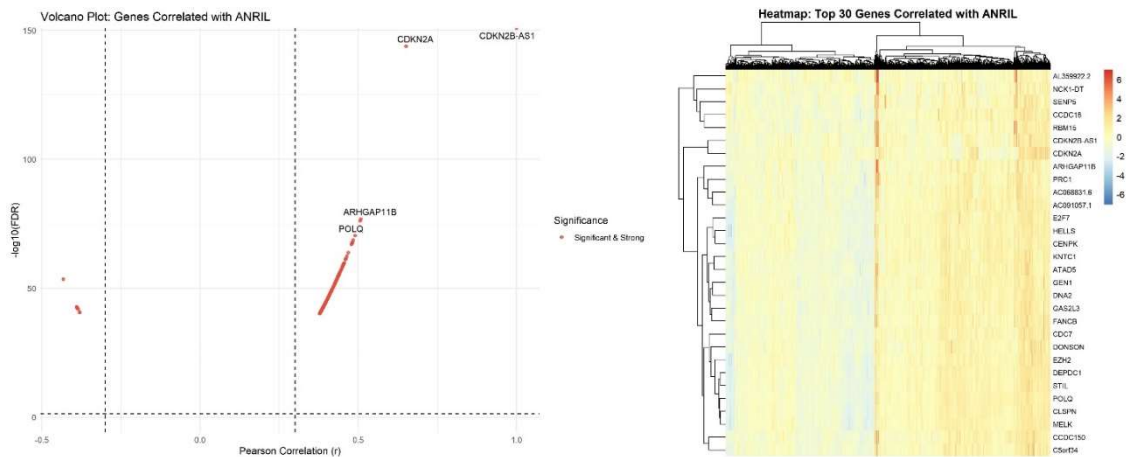


Figure 5. (A) Volcano plot of genome-wide correlations with ANRIL ($|r| > 0.3$ and $FDR < 0.05$ highlighted in red). (B) Heatmap of the top 30 positively correlated genes (row-scaled VST expression).

survival in the high-risk group, defined as patients with both high ANRIL and high IL-6 levels, compared with the other groups; however, this difference did not reach statistical significance (log-rank $P = 0.052$; **Figure 4C**).

3.8. Co-expression network and genes associated with ANRIL

Genome-wide correlation analysis identified several genes showing strong positive correlations with ANRIL expression (**Figure 5A**). A heatmap of the top 30 ANRIL-associated genes revealed two distinct expression clusters across the tumor samples, with ANRIL expression showing a pattern concordant with the high-expression cluster (**Figure 5B**).

3.9 .Functional Enrichment of ANRIL-Associated Genes

Functional enrichment analysis of the ANRIL-associated genes revealed significant enrichment in pathways related to the cell cycle and mitosis. The top Gene Ontology (GO) biological processes included sister chromatid separation in mitosis, microtubule cytoskeleton organization involved in mitosis, mitotic spindle organization, and mitotic nuclear division (**Table 5**). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis predominantly identified pathways related to the cell cycle (**Table 6**). Reactome pathway analysis similarly highlighted processes involved in the cell cycle, mitosis, and cell-cycle checkpoints (**Table 7**). Collectively, these findings suggest that ANRIL expression is associated with genes and biological pathways involved in cell-cycle regulation, mitosis, and cell proliferation.

3.10. ANRIL and IL6 expression by PAM50 molecular subtype

IL6 expression was lower in the Luminal subtypes than in the Normal-like and Basal-like subtypes (**Figure 6A**). In contrast, ANRIL expression was significantly higher in the Basal-like and Luminal B subtypes than in the Luminal A and HER2 subtypes ($P < 0.001$; **Figure 6B**). This expression pattern is consistent with the greater proliferative characteristics observed in more aggressive breast cancer molecular subtypes and may contribute to the observed association between ANRIL-IL6 expression patterns and prognosis.

3.11. Correlation analysis with oxidative stress-related genes

To investigate the association between ANRIL (CDKN2B-AS1; antisense non-coding RNA in the INK4 locus)

expression and oxidative stress-related genes, correlations between ANRIL and 10 key oxidative stress-related genes, including NOX4 (NADPH oxidase 4), GPX1 (glutathione peroxidase 1), GPX4 (glutathione peroxidase 4), SOD1 (superoxide dismutase 1), SOD2 (superoxide dismutase 2), HMOX1 (heme oxygenase 1), TXN (thioredoxin), TXNRD1 (thioredoxin reductase 1), PRDX1 (peroxiredoxin 1), and CAT (catalase), were assessed using the GEPIA2 platform based on TCGA-BRCA data. No statistically significant correlation was observed between ANRIL expression and any of the evaluated oxidative stress-related genes (all Pearson $|r| < 0.2$ and $P > 0.05$; data not shown). These findings indicate that ANRIL expression was not significantly associated with the expression of the selected oxidative stress-related genes in the analyzed TCGA-BRCA dataset. Therefore, the potential relationship between ANRIL and oxidative stress may involve mechanisms not captured by direct gene-expression correlations and warrants further investigation.

4. DISCUSSION

The present study investigated the expression profile of lncRNA ANRIL in breast cancer patients and evaluated its relationship with oxidative stress biomarkers and inflammatory status. The findings demonstrated a significant dysregulation of circulating ANRIL expression in breast cancer patients compared with healthy controls, accompanied by increased oxidative stress, impaired antioxidant defense, and elevated plasma IL-6 levels. Furthermore, ROC curve analysis revealed a high diagnostic performance for ANRIL expression, suggesting its potential utility as a noninvasive biomarker for breast cancer detection.

Importantly, no significant differences were observed between the patient and control groups regarding BMI, reducing the likelihood that oxidative stress alterations were influenced by obesity-related metabolic factors. Although the mean age appeared higher in the cancer group, statistical analysis did not demonstrate a significant difference between groups. Therefore, the observed molecular and biochemical alterations may primarily reflect disease-related mechanisms rather than demographic confounding variables.

Accumulating evidence suggests that ANRIL functions as an important regulator in breast cancer biology. Previous studies have demonstrated that ANRIL expression is significantly increased in triple-negative breast cancer (TNBC) tissues and cell lines and is associated with poor prognosis, enhanced proliferation, migration, invasion, and resistance to apoptosis (20). Mechanistically, ANRIL has

Table 5. Top Biological Process

Term	Adjusted.P.value	Genes
Mitotic Sister Chromatid Segregation (GO:0000070)	2.69E-18	NCAPG2; KIF14; NCAPG; KIF23; KIF11; NDC80; SGO1; TPX2; CENPE; KIF18A; KIF18B; ESPL1; CENPI; CENPK; KIF2C; CEP55; DLGAP5
Microtubule Cytoskeleton Organization Involved in Mitosis (GO:1902850)	6.83E-11	GPSM2; CENPE; STIL; ESPL1; KIF4A; NUF2; TTK; KIF11; DLGAP5; NDC80
Mitotic Spindle Organization (GO:0007052)	6.83E-11	SGO1; KIF18A; KIF18B; ESPL1; CENPI; NCAPG2; CENPK; NDC80
Sister Chromatid Segregation (GO:0000819)	5.63E-10	CD2; FCRL3; TBX21; CRTAM; IKZF3; CCR2
Mitotic Nuclear Division (GO:0140014)	5.11E-09	SGO1; KIF18A; KIF18B; ESPL1; CENPI; NCAPG2; CENPK; NDC80

Table 6. Top KEGG 2021 Human

Term	Adjusted.P.value	Genes
Cell cycle	5.72E-11	PTTG1; CCNE2; ESPL1; CCNE1; CDKN2A; BUB1B; E2F2; CDC7; TTK; SKP2; CDC25C; BUB1
Oocyte meiosis	6.27E-06	SGO1; PTTG1; CCNE2; ESPL1; CCNE1; CDC25C; FBXO43; BUB1
Viral carcinogenesis	1.26E-04	H2BC13; CCNE2; CCNE1; CDKN2A; H4C2; SKP2; H4C3; H2BC17
Homologous recombination	5.52E-04	BLM; BRIP1; XRCC2; RAD54L
Systemic lupus erythematosus	5.52E-04	H2AC12; H2BC13; H4C2; H3C2; H4C3; H2BC17

Table 7. Top Reactome 2022

Term	Adjusted.P.value	Genes
Cell Cycle R-HSA-1640170	7.34E-31	PIF1; ERCC6L; BLM; NCAPG2; HJURP; BUB1B; NCAPG; MCM10; HMMR; CENPA; SKA1; BRIP1; PTTG1; NUF2; E2F2; CLSPN; SKP2; GTSE1; BUB1; CDKN2A; CDC7; KIF23; KNL1; CDC25C; H2BC17; NDC80; SGO1; TPX2; H2BC13; CENPE; KIF18A; CENPF; CCNE2; ESPL1; CCNE1; CENPI; CENPK; KIF2C; DNA2; KIF20A
Cell Cycle, Mitotic R-HSA-69278	4.27E-28	ERCC6L; NCAPG2; BUB1B; NCAPG; MCM10; HMMR; CENPA; SKA1; PTTG1; NUF2; E2F2; SKP2; GTSE1; BUB1; CDKN2A; CDC7; KIF23; KNL1; CDC25C; H2BC17; NDC80; SGO1; TPX2; H2BC13; CENPE; KIF18A; CENPF; CCNE2; ESPL1; CCNE1; CENPI; CENPK; KIF2C; DNA2; KIF20A
Cell Cycle Checkpoints R-HSA-69620	2.40E-27	ERCC6L; BLM; BUB1B; MCM10; CENPA; SKA1; BRIP1; NUF2; CLSPN; GTSE1; BUB1; CDKN2A; CDC7; KNL1; CDC25C; H2BC17; NDC80; SGO1; H2BC13; CENPE; KIF18A; CENPF; CCNE2; CCNE1; CENPI; CENPK; KIF2C; DNA2
RHO GTPase Effectors R-HSA-195258	1.29E-17	ERCC6L; KIF14; BUB1B; KNL1; CDC25C; IQGAP3; H2BC17; CENPA; SKA1; NDC80; SGO1; H2BC13; CENPE; KIF18A; CENPF; DIAPH3; CENPI; NUF2; CENPK; KIF2C; BUB1
Unattached Kinetochores Signal Amplification Via A MAD2 Inhibitory Signal R-HSA-141444	3.30E-17	ERCC6L; BUB1B; KNL1; CENPA; SKA1; NDC80; SGO1; CENPE; CENPF; KIF18A; CENPI; NUF2; CENPK; KIF2C; BUB1

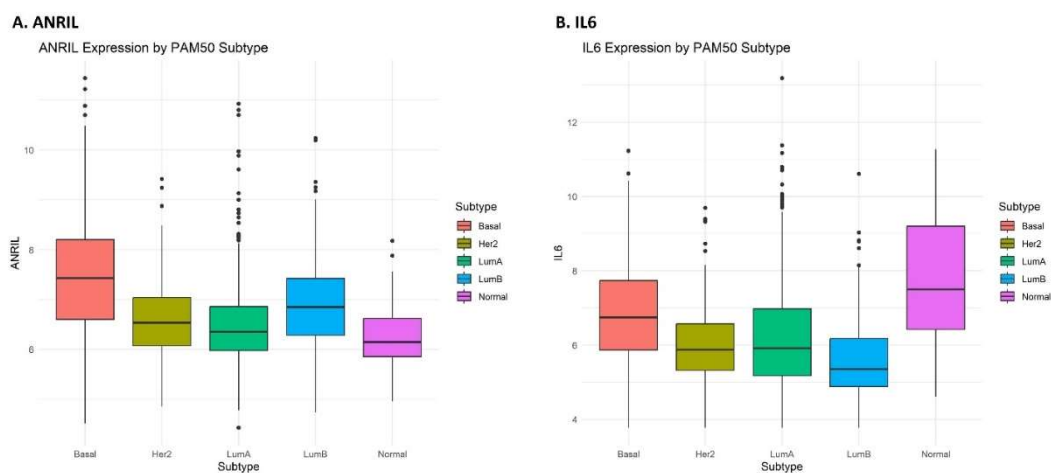


Figure 6. (A) ANRIL and (B) IL-6 Expression by PAM50 Subtype

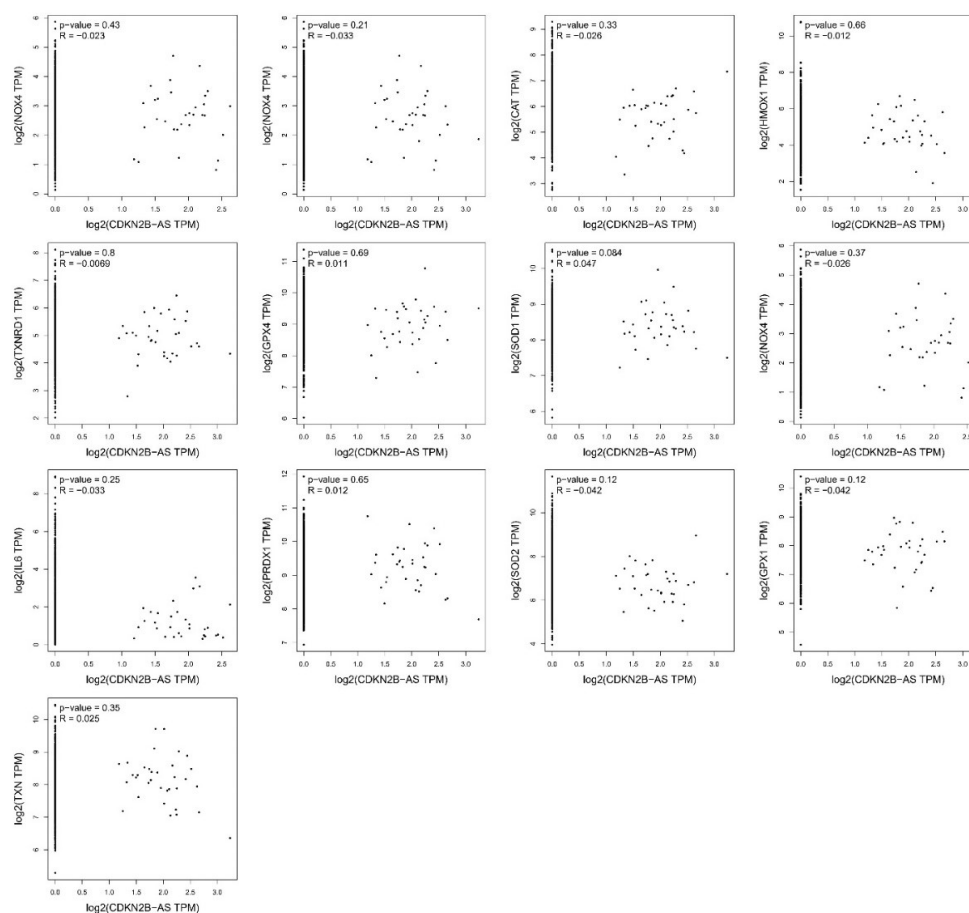


Figure 7. Correlation analysis of ANRIL (CDKN2B-AS1) expression with key oxidative stress-related genes in the TCGA-BRCA cohort using GEPIA2. Pairwise scatter plots of ANRIL expression (x-axis: log₂(CDKN2B-AS1 TPM)) against NOX4, CAT, HMOX1, TXNRD1, GPX4, SOD1, NOX4, GPX1, PRDX1, SOD2, and TXN (top-left to bottom-right). No significant correlations were observed in any pair ($|R|$ ranging from 0.0026 to 0.047, all p-values > 0.05).

been shown to act as a competing endogenous RNA (ceRNA) by sponging miR-199a, thereby modulating downstream oncogenic signaling pathways involved in tumor progression and survival (20). Functional studies further demonstrated that ANRIL silencing suppresses proliferation and induces apoptosis in TNBC cells, supporting its involvement in breast cancer pathogenesis (20).

Interestingly, in contrast to several tissue-based studies reporting ANRIL overexpression in breast tumors, the present study demonstrated a significant reduction in circulating ANRIL expression in peripheral blood leukocytes of breast cancer patients. This apparent discrepancy may reflect tissue-specific and circulation-specific expression patterns of ANRIL. Previous investigations have shown that expression profiles of several lncRNAs differ substantially between tumor tissue, serum, plasma, and immune cells. Therefore, reduced ANRIL expression in circulating leukocytes may represent systemic immune dysregulation or altered inflammatory responses associated with cancer progression rather than direct tumor-cell expression. Consequently, circulating ANRIL may provide biological information distinct from intratumoral ANRIL expression. Consistent with the current findings, previous clinical studies have identified circulating lncRNAs as promising biomarkers in breast cancer. A study investigating serum levels of ANRIL, TUG1, UCA1, and HIT in patients with invasive ductal carcinoma demonstrated significant dysregulation of these lncRNAs and reported associations with tumor stage, hormone receptor status, and metastatic progression (19). These findings suggested that ANRIL may serve as a potential biomarker for diagnosis and disease monitoring (19). Similarly, the ROC analysis in the present study demonstrated excellent discriminatory ability for ANRIL expression, supporting its possible clinical application as a diagnostic biomarker in breast cancer patients.

The bioinformatic findings reported in previous studies further support the biological relevance of ANRIL in breast cancer progression. Deng (2022) demonstrated that CDKN2B-AS1 (ANRIL) co-expressed genes are strongly enriched in pathways associated with mitosis, chromosomal segregation, and cell-cycle progression. Moreover, ANRIL has been associated with the regulation of proliferation-related genes including CDK1, CDC25A, and SPC25, suggesting that this lncRNA may contribute to aggressive tumor behaviour and poor prognosis. Increased ANRIL expression has also been linked to high-risk PAM50 molecular subtypes such as Basal-like and Luminal B breast

cancers, both characterized by high proliferative activity and unfavourable clinical outcomes (21, 22).

Inflammation is another major component of ANRIL-associated molecular signaling. Previous studies demonstrated that ANRIL participates in regulation of the NF- κ B pathway, a central mediator of inflammation-associated carcinogenesis (23). ANRIL expression can be induced by inflammatory cytokines such as TNF- α through NF- κ B activation, while ANRIL itself modulates inflammatory mediators including IL-6 and IL-8 via interaction with the transcription factor YY1 (23). In the present study, plasma IL-6 levels were significantly elevated in breast cancer patients compared with healthy controls, indicating activation of systemic inflammatory pathways in the disease state.

Elevated IL-6 levels have been strongly associated with tumor progression, angiogenesis, immune evasion, and metastasis in breast cancer. A recent systematic review and meta-analysis identified IL-6 as an unfavourable prognostic marker in breast cancer patients (24). However, despite significant elevation of IL-6 levels in the present study, no significant correlation was observed between ANRIL expression and IL-6 concentrations. This finding may indicate that ANRIL and IL-6 represent relatively independent molecular axes involved in proliferation and inflammation, respectively. Similarly, Hu (2024) suggested that combining proliferation-associated lncRNAs with inflammatory biomarkers may improve prognostic models even in the absence of direct molecular correlations (25).

A major finding of the present study was the presence of pronounced oxidative stress in breast cancer patients. MDA levels were significantly elevated, whereas TAC and SOD levels were significantly reduced in the cancer group compared with controls. Increased MDA levels indicate enhanced lipid peroxidation and oxidative cellular damage, while decreased TAC and SOD levels reflect impaired antioxidant defense capacity. These findings collectively suggest substantial oxidative imbalance in breast cancer patients.

Oxidative stress is recognized as a critical contributor to carcinogenesis through induction of DNA damage, mitochondrial dysfunction, genomic instability, and dysregulation of intracellular signalling pathways. Cancer cells commonly exhibit excessive reactive oxygen species (ROS) production accompanied by impaired antioxidant systems, facilitating tumor progression and survival. In the present study, a significant positive correlation was identified between TAC and SOD in the cancer group, suggesting coordinated regulation of enzymatic and

nonenzymatic antioxidant defense systems during disease progression.

Previous studies also suggest a possible association between ANRIL and oxidative stress pathways. Under hyperglycemic conditions, increased ANRIL expression in renal podocytes was associated with enhanced oxidative stress, inflammation, and apoptosis, whereas ANRIL silencing reduced these pathological processes through modulation of MME expression (18). These findings indicate that ANRIL may participate indirectly in oxidative stress-related molecular pathways. In the current study, although no statistically significant correlations were observed between ANRIL and oxidative stress markers, a positive trend between ANRIL and MDA levels was identified in cancer patients. This observation may suggest a possible interaction between ANRIL dysregulation and oxidative damage that warrants further investigation in larger cohorts.

Interestingly, previous bioinformatic analyses demonstrated limited direct associations between ANRIL and classical oxidative stress genes such as SOD1, SOD2, GPX1, GPX4, CAT, TXN, and NOX4. Recent reviews therefore propose that the principal functions of ANRIL are more closely related to epigenetic regulation, proliferation, migration, and inflammatory signalling rather than direct antioxidant regulation (26). Accordingly, the oxidative stress alterations observed in the present study may represent secondary consequences of tumor progression and chronic inflammation rather than direct ANRIL-mediated antioxidant dysregulation.

Overall, the findings of the present study suggest that ANRIL may contribute to breast cancer pathogenesis through interactions with proliferative, inflammatory, and oxidative stress-related pathways. The significant dysregulation of circulating ANRIL expression together with its favorable diagnostic performance highlights its potential value as a novel biomarker in breast cancer. Moreover, integration of ANRIL with inflammatory biomarkers such as IL-6 may improve future prognostic and diagnostic models.

Several limitations of the present study should be acknowledged. First, the relatively small sample size may have limited the statistical power for detecting weaker correlations between ANRIL expression and oxidative stress markers. Second, ANRIL expression was evaluated only in peripheral blood leukocytes rather than tumor tissue, preventing direct comparison between circulating and intratumoral expression patterns. Third, the cross-sectional design of the study limits the ability to establish causal relationships between ANRIL dysregulation, inflammation, and oxidative stress. Additionally, functional molecular

experiments were not performed to clarify the mechanistic role of ANRIL in breast cancer progression.

Future studies with larger patient populations and multicenter validation are required to confirm the diagnostic and prognostic value of circulating ANRIL in breast cancer. Simultaneous evaluation of ANRIL expression in tumor tissue, plasma, and immune cells may help clarify tissue-specific expression patterns and biological functions. Furthermore, mechanistic investigations focusing on ANRIL-mediated regulation of inflammatory and oxidative stress pathways could improve understanding of its role in breast cancer pathogenesis. Integration of ANRIL with inflammatory cytokines, oxidative stress biomarkers, and genomic signatures may also contribute to the development of more accurate molecular panels for early diagnosis, prognosis, and personalized therapeutic strategies in breast cancer.

5. CONCLUSION

This study demonstrated significant dysregulation of circulating lncRNA ANRIL expression in breast cancer patients, accompanied by increased oxidative stress and inflammation. Elevated MDA and IL-6 levels together with reduced TAC and SOD activities indicated enhanced lipid peroxidation, impaired antioxidant defense, and activation of inflammatory pathways in breast cancer. ROC curve analysis also revealed favorable diagnostic performance for ANRIL expression, suggesting its potential utility as a non-invasive biomarker. Although no significant direct correlation was observed between ANRIL expression and oxidative stress markers or IL-6 levels, the findings suggest that ANRIL may participate indirectly in molecular pathways associated with tumor progression and inflammatory responses. Overall, ANRIL, together with oxidative stress and inflammatory biomarkers, may contribute to breast cancer pathogenesis and could represent a promising target for future diagnostic and therapeutic strategies. Further large-scale and mechanistic studies are needed to validate these findings and clarify the precise biological role of ANRIL in breast cancer.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Ethical statement

This study was approved by the Ethics Committee of Mazandaran University of Medical Sciences (IR.MAZUMS.REC.1404.565). All ethical principles and relevant guidelines were observed throughout the study.

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