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Immune-related lncRNA LINC00944 responds to variations in ADAR1 levels and it is associated with breast cancer prognosis

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ABSTRACT

Aims: Breast cancer is one of the leading causes of woman deaths worldwide, being a major public health problem. It has been reported that the expression of the RNA-editing enzyme Adenosine Deaminase Acting on RNAs 1 (ADAR1) is upregulated in breast cancer, predicting poor prognosis in patients. A few reports in literature examine ADAR1 and long non-coding RNAs (lncRNAs) interplay in cancer and suggest key roles in cancer-related pathways. This study aimed to investigate whether ADAR1 could alter the expression levels of lncRNAs and explore how those changes are related to breast cancer biology.

Main methods: ADAR1 overexpression and knockdown studies were performed in breast cancer cell lines to analyze the effects over lncRNAs expression. Guilt-by-Association correlation analysis of the TCGA-BRCA cohort was performed to predict the function of the lncRNA LINC00944.

Key findings: Here, we show that LINC00944 is responsive to ADAR1 up- and downregulation in breast cancer cells. We found that LINC00944 expression has a strong relationship with immune signaling pathways. Further assessment of the TCGA-BRCA cohort showed that LINC00944 expression was positively correlated to tumor-infiltrating T lymphocytes and pro-apoptotic markers. Moreover, we found that LINC00944 expression was correlated to the age at diagnosis, tumor size, and estrogen and progesterone receptor expression. Finally, we show that low expression of LINC00944 is correlated to poor prognosis in breast cancer patients.

Significance: Our study provides further evidence of the effect of ADAR1 over lncRNA expression levels, and on the participation of LINC00944 in breast cancer, suggesting to further investigate its potential role as prognostic biomarker.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related deaths in women [1]. The Global Cancer Observatory (GLOBOCAN) projected 2,088,849 cases in 2018, worldwide, which represented 11.6% of total cancer cases [1]. According to the American Cancer Society, in 2020, there will be diagnosed about 276,480 cases of invasive breast cancer and about 48,530 new cases of carcinoma in situ only in the United States, while about 42,170 women will die from breast cancer. These alarming numbers highlight the extent of this major public health problem and prove the need for broader knowledge in basic research, diagnosis, and treatment of breast cancer.

The Adenosine Deaminase Acting on RNAs (ADAR) family of proteins is integrated by three members, ADAR1, ADAR2, and ADAR3, with

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ADAR1 indispensable for life in mammals [2,3]. The canonical function of ADAR1 and ADAR2 is the deamination of Adenine-to-Inosine in double-stranded RNAs (dsRNAs), in a process known as A-to-I RNA editing [2]. ADAR1 RNA editing has been strongly associated with cancer progression by promoting proliferation, invasion, migration, or drug sensitivity in several cancer types, including breast cancer [4–6], hepatocellular carcinoma [7], cervical cancer [8,9], gastric cancer [10], non-small-cell lung cancer [11], esophageal squamous cell carcinoma [12], and thyroid cancer [13], among others.

Elevated *ADAR1* expression has been reported in breast cancer [4,14,15], predicting poor overall survival in patients [6,16]. We and others have shown that modulation of *ADAR1* expression levels leads to expression changes in coding and non-coding RNAs, and that is an important factor in cancer biology [6,17,18]. Among non-coding RNAs, long non-coding RNAs (lncRNAs) have emerged as central players in cancer development and progression [19], and have been proposed as prognostic biomarkers in several cancer types [20–22]. However, few reports regarding the ADAR1 effect over lncRNAs expression and/or function are found in the literature [23–26]. For instance, it has been shown in human prostate cancer that the antisense lncRNA PCA3 can promote malignant cell growth by controlling the expression levels of the tumor suppressor PRUNE2, via the formation of an RNA duplex and the nuclear sequestration induced by ADAR1 A-to-I editing [23]. In pancreatic cancer, it has been proved that the antisense lncRNA of glutaminase (GLS-AS) can repress glutaminase (GLS) expression through an ADAR1/Dicer-dependent RNA interference mechanism, thus impairing the metabolic reprogramming mediated by GLS in cancer cells. Accordingly, low expression of GLS-AS was associated with poor clinical outcomes [24].

As a result of the prior research, the emerging picture of ADAR1-lncRNAs interplay in cancer biology prompted us to further investigate the effect of ADAR1 over lncRNAs expression in breast cancer. To this end, we examined RNA-seq data on the MDA-MB-231 cell line overexpressing ADAR1 to look for differentially expressed lncRNAs. Next, ADAR1 gain-of-function and loss-of-function approaches were used to validate expression changes, finding that the long intergenic non-coding RNA LINC00944 presented very consistent expression changes. By using the Guilt-by-Association method, we investigated the LINC00944 function. Interrogating the breast cancer cohort of The Cancer Genome Atlas (TCGA-BRCA) enabled us to correlate LINC00944 expression to tumor-infiltrating lymphocytes (TILs) and apoptotic markers. Finally, we were able to correlate LINC00944 expression to clinicopathological parameters and survival outcomes in breast cancer patients.

2. Methods

2.1. Cell culture

MDA-MB-231(ATCC, Cat# HTB-26) and MDA-MB-436 (ATCC, Cat# HTB-130) human cell lines were obtained from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle medium (DMEM)-F12 (HyClone, Cat# SH30261.01) supplemented with 10% fetal bovine serum (FBS) (Corning, Cat# 35-010-CV) and 100 U/ml penicillin and streptomycin solution (Corning, Cat# 30-002C1). All cell lines were maintained at sub-confluent densities at 37 °C in a 5% CO_2 humidified incubator.

2.2. Adenovirus transduction

ADAR1 overexpression was induced in the breast cancer cell line MDA-MB-231 by using an adenovirus vector carrying the short isoform of the human ADAR1 DNA sequence (ADAR1-p110) (NM_001025107.2) (ADAR1 OE condition) (Vector Builder, pAV[Exp]-CMV>hADAR [NM_001025107.2]*/3xFLAG). A GFP sequence was used in the control condition (Mock condition) (Vector Builder, pAV[Exp]-CMV>EGFP). For each condition and biological replicate, 500,000 cells were plated in a 60 mm dish. After 24 h, the adenovirus particles were transduced at a multiplicity of infection (MOI) of 200, and the expression was allowed for additional 48 h.

2.3. Small interfering RNAs (siRNAs) transfection

The siRNAs transfections in the MDA-MB-231 and MDA-MB-436 cell lines were carried out using Lipofectamine 3000 Transfection Reagent (Thermo Scientific, Cat# L300000) according to the manufacturer's directions. Briefly, 500,000 cells were plated in a 60 mm dish. After 24 h, control (Cell Signaling, Cat# 6568S) or ADAR1 (Ambion, Cat# AM51331) siRNAs were transfected at a final concentration of 20 nM and incubated for additional 48 h.

2.4. RNA isolation and DNase treatment

Total RNA was isolated using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Cat# R6834), according to the manufacturer's instructions. Samples were treated with RNase-free DNase Set I (Omega Bio-Tek, Cat# E1091) for DNA removal, as the manufacturer recommended. Concentration and quality RNAs were measured in the Take 3 - Cytation 3 Instrument (Biotek). Only suitable samples were used for downstream applications. All RNAs obtained were stored at −80 °C.

2.5. cDNA synthesis

The cDNA was synthesized by using the AffinityScript cDNA Synthesis Kit (Agilent Technologies, Cat# 600559), according to the manufacturer's protocol. One μg of RNA was used as a template in each reverse transcription reaction, and a mixture of oligo-dT and random primers was used to improve efficiency. All cDNA products were stored at −20 °C.

2.6. RT-qPCR

Real-time PCR reactions were performed using an Eco Real-Time PCR System (Illumina, Cat# EC-900-1001) and Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Cat# 600828). Reactions were prepared and performed according to the manufacturer's instructions. For the detection of mRNAs and lncRNAs, 35 and 40 cycles were used, respectively. β-actin served as an internal control. Relative RNA expression was calculated using the comparative cycle threshold (Ct) ($2^{-\Delta\Delta Ct}$) method. All primers were tested for efficiency, and only primers with amplification efficiencies ranging from 90% to 110% were used. Primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) [27] and synthesized by Integrated DNA Technologies (IDT).

2.7. RESSqPCR

RNA Editing Site-Specific-qPCR (RESS-qPCR) was performed as described in Crews et al., 2015 [28]. Briefly, for detecting each RNA editing site, two sets of primers were used, one pair for detecting WT transcript, and one pair detecting the edited transcript. cDNA synthesis and RT-qPCR were performed as described previously. Relative RNA editing ratios (Relative Edited/WT RNA) were calculated using the comparative cycle threshold (Ct) [$2^{-(Ct\ Edit\ -\ Ct\ WT)}$] [28].

2.8. Protein extraction and Western blot

Cells were lysed with RIPA lysis buffer (Thermo Scientific, Cat# 89900) and protease inhibitors (Thermo Scientific, Cat# 1861284) following the manufacturer's protocol. Cell homogenates were separated by a 10% SDS-PAGE (Bio-Rad Laboratories, Cat# 4561034). Anti-ADAR1 (1:2000 rabbit polyclonal, Abcam, Cat# ab168809) and anti-

β-actin (1:5000 Sigma-Aldrich, A1978) primary antibodies were probed overnight at 4 °C. HRP-linked secondary antibodies (1:5000 dilution) were incubated 1.5 h at room temperature, and blots were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Cat# 34579) in the ChemiScope3500 Mini chemiluminescence imaging system (Clinx Science Instruments). Western blot densitometry was performed using Image Studio Lite Software v5.2 (LI-COR) and normalized by β-actin.

2.9. RIP-qPCR

ADAR1 RNA-immunoprecipitation was performed as previously described in Hendrickson et al., 2016 [29]. Briefly, 5×10^6 cells/ml were crosslinked using formaldehyde (Thermo Scientific, Cat# 28908) to a final concentration of 0.1%. Cell were resuspended in 1 ml of RIPA lysis buffer with protease inhibitors and RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, Cat# 10777-019). Cell lysates were incubated with 4 μg of ADAR1 antibody (Abcam, Cat# ab168809). Dynabeads Protein G (Thermo Scientific, Cat # 10003D) were added and rotated at 4 °C. Beads were resuspended in RNase-free water, 3× reverse-crosslinking buffer, Proteinase K (Invitrogen, Cat# 25530049), and RNaseOUT Recombinant Ribonuclease Inhibitor. Protein degradation and reverse-crosslinking were performed for 1 h at 42 °C plus 1 h at 55 °C. 1 ml of TRIzol Reagent (Invitrogen, Cat# 15596026) was used for RNA isolation, and RT-qPCR was performed as mentioned above.

2.10. Analysis of differentially expressed lncRNAs

Expression data were obtained from Sagredo et al., 2020 [30]. Differential expression analysis between MDA-MB-231 Mock and ADAR1 overexpression conditions was performed using DEseq2 software following standard recommendations [31]. A False Discovery Rate (FDR) ≤ 0.05 was considered statistically significant, and no fold change cutoff was considered given that observed changes in lncRNAs expression levels were modest.

2.11. Kaplan-Meier survival curves

Overall Survival (OS) and Relapse-free Survival (RFS) curves were performed using the web-based Kaplan-Meier Plotter (https://kmplot.com/analysis/) [32] on the breast cancer dataset. Triple-negative breast cancer curves were analyzed by selecting estrogen receptor negative, progesterone receptor negative, and HER2 negative patients. Gene symbol: LINC00944, Affymetrix ID: 1560573_at. A *Log-rank* $p \leq 0.05$ was considered statistically significant.

2.12. Gene expression data retrieval

Gene expression (FPKM-UQ) and clinical data were obtained from The Cancer Genome Atlas (TCGA) Project (https://www.cancer.gov/tcga) through the Genomic Data Commons (GDC) data portal (https://portal.gdc.cancer.gov/) [33]. TCGA studies retrieved: Breast invasive carcinoma (BRCA), Colon adenocarcinoma (COAD), Lung adenocarcinoma (LUAD), and Testicular Germ Cell Tumors (TGCT).

2.13. Protein levels data retrieval

Protein levels based on Reverse Phase Protein Array (RPPA) data were obtained from the Breast Invasive Carcinoma study [34] deposited in the cBioPortal for Cancer Genomics (http://cbioportal.org) [35,36].

2.14. Guilt-by-association analysis

LINC00944 expression was correlated to protein-coding genes (GRCh38) expression in normal and tumor samples by Pearson Correlation. The *p*-value was adjusted using the Benjamini-Hochberg method, and an FDR ≤ 0.05 was considered statistically significant. Correlations with Pearson coefficient (r) $\leq |0.3|$ were taken for computing the overlap against Hallmark gene sets collection [37] using the Gene Set Enrichment Analysis (GSEA) online tool (https://www.gsea-msigdb.org/gsea/index.jsp) [38]. An FDR ≤ 0.05 was considered statistically significant.

2.15. LINC00944 expression and clinicopathological parameters correlation

LINC00944 expression and clinical data from TCGA-BRCA were obtained as mentioned above. Patients were classified based on their LINC00944 z-score. Upper (High LINC00944 expression group) and lower (Low LINC00944 expression group) quartiles were chosen for further comparison (n=250). Data were arranged in 2×2 contingency tables, and the *Fisher's exact test* was run for the comparison. A p-value ≤ 0.05 was considered statistically significant.

2.16. Statistical analyses

R Software v3.6 (R Core Team, 2020) was used for data management. Statistical tests were performed in R Software v3.6 and GraphPad Prism v8.3 (GraphPad Software, La Jolla, CA, USA). A two-tailed *Student's t-test* was used to establish differences between two groups in RT-qPCR, and each analysis was performed in 5–6 independent experiments. For non-parametric data, the *Mann-Whitney test* was used in two-group comparisons. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. ADAR1 overexpression induces differential expression of lncRNAs

To assess the effect of *ADAR1* overexpression over lncRNAs expression levels, we examined our previously reported RNA-seq data on the breast cancer cell line MDA-MB-231 overexpressing ADAR1 [30]. The ADAR1 overexpression resulted in the upregulation of 24 lncRNAs and the downregulation of 17 lncRNAs (p-adj ≤ 0.05) (Fig. 1A and Supplementary Table 1). According to GENCODE biotype annotation, differentially expressed lncRNAs were composed of 14 antisense RNAs (ASRNAs), 18 long intergenic non-coding RNAs (lincRNAs), 7 processed transcripts, and 2 sense intronic RNAs (Fig. 1A).

In order to validate the observed expression changes in lncRNAs, we overexpressed *ADAR1* on the MDA-MB-231 cell line by using an adenovirus vector and assessed lncRNAs expression using RT-qPCR. Two downregulated lncRNAs, LINC00944 and APCDD1L-AS1, and two upregulated lncRNAs, LINC01003 and H1FX-AS1, were taken for this purpose. Controls for ADAR1 mRNA expression, protein levels, and function upregulation were performed (Supplementary Fig. 1). As shown in Fig. 1B, we validated the expression changes for LINC00944 ($p = 0.0003$, *Student's t-test*), APCDD1L-AS1 ($p = 0.0327$, *Student's t-test*) and LINC01003 ($p = 0.001$, *Student's t-test*), but H1FX-AS1 did not reach a significant difference ($p = 0.055$, *Student's t-test*) (Fig. 1B).

Aiming to corroborate our findings in a second breast cancer cell line, we attempted to overexpress *ADAR1* in MDA-MB-436 cells. We were not able to obtain viable MDA-MB-436 ADAR1-overexpressing cells, perhaps because MDA-MB-436 has high intrinsic levels of ADAR1 compared to MDA-MB-231 and other breast cancer cell lines [39] (Supplementary Fig. 2).

3.2. ADAR1 knockdown reverted the effect in LINC00944 expression levels

To further examine whether ADAR1 could alter the expression levels of lncRNAs in breast cancer, we investigated the effect of *ADAR1* loss-of-function in the MDA-MB-231 cell line using a siRNA (siADAR1). Controls for ADAR1 mRNA expression, protein levels, and function

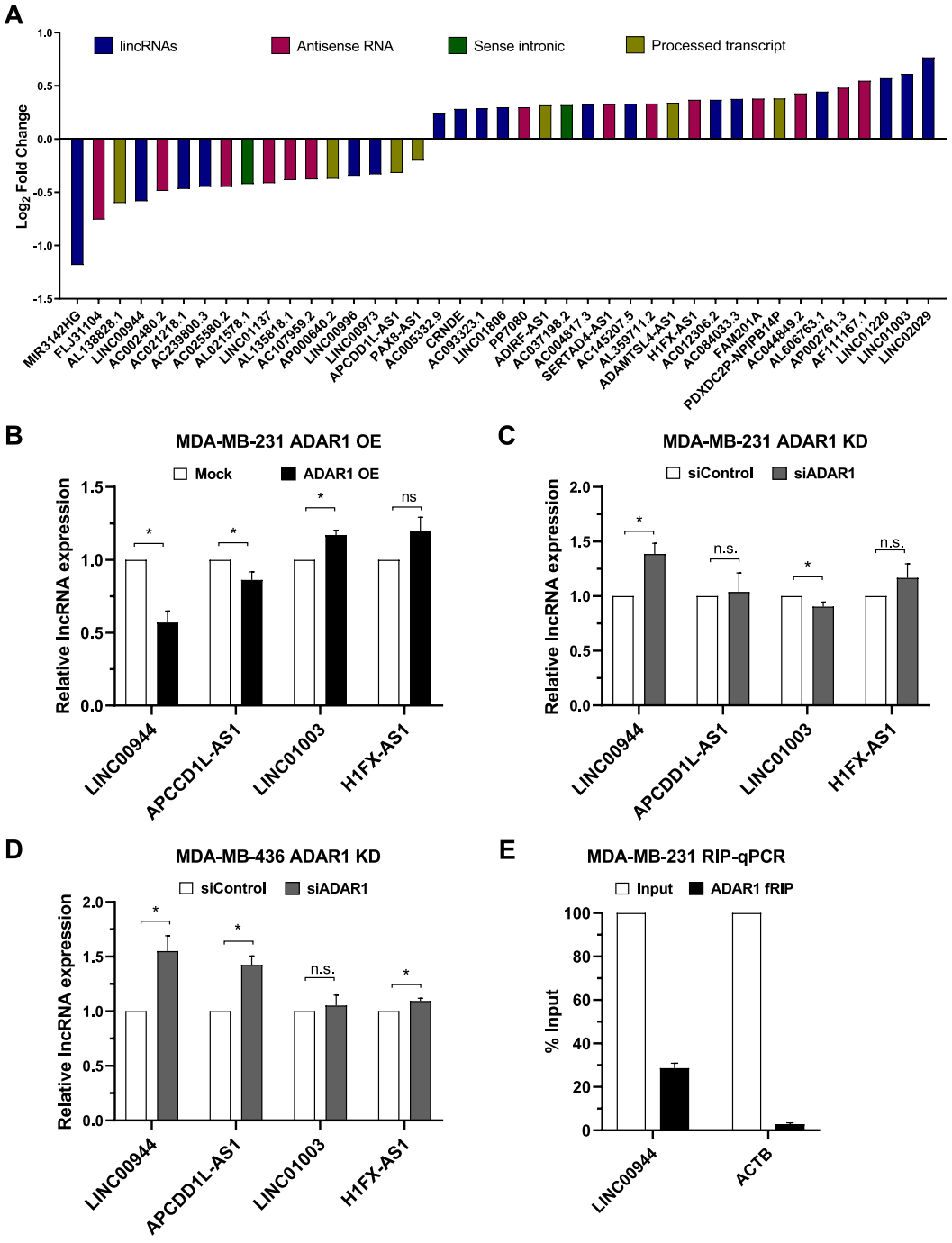

Fig. 1. ADAR1 overexpression effects over lncRNAs expression levels in breast cancer cell lines. (A) Differentially expressed lncRNAs in MDA-MB-231 ADAR1 OE. *LincRNAs* are depicted in blue, *antisense* in pink, *sense intronic* in green and *processed transcripts* in yellow. (B) RT-qPCR. Experimental validation of LINC00944 ($p = 0.0003$), APCDD1L-AS1 ($p = 0.0327$), LINC01003 ($p = 0.001$) and H1FX-AS1 ($p = 0.055$) expression changes in MDA-MB-231 ADAR1 OE. (C) RT-qPCR. Assessment of LINC00944 ($p = 0.0029$), APCDD1L-AS1 ($p = 0.835$), LINC01003 ($p = 0.0441$) and H1FX-AS1 ($p = 0.2212$) expression levels in MDA-MB-231-ADAR1 KD and (D) MDA-MB-436-ADAR1 KD cell lines (LINC00944, $p = 0.0029$; APCDD1L-AS1, $p = 0.0004$; LINC01003, $p = 0.5977$; H1FX-AS1, $p = 0.0031$). Expression values were calculated relative to control condition using $2^{-(\Delta\Delta Ct)}$. β-actin was used as internal control. Data are shown as mean, and error bars represent ± SEM of 5–6 biological replicates. Data were analyzed using unpaired, two-tailed *Student's t-test*. A *p*-value ≤ 0.05 was considered statistically significant. '*ns*' indicates no significant difference between compared groups. (E) RIP-qPCR. ADAR1 RNA immunoprecipitation followed by RT-qPCR in MDA-MB-231 cells. Values are relative to input sample. *ACTB* was used as negative control. *N*=2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

downregulation were performed (Supplementary Fig. 3). Interestingly, ADAR1 downregulation resulted in the upregulation of LINC00944 ($p = 0.0029$, *Student's t-test*), and the downregulation of LINC01003 ($p = 0.0441$, *Student's t-test*). However, we did not observe changes in APCDD1L-AS1 or H1FX-AS1 expression levels ($p = 0.835$ and $p = 0.2212$ respectively, *Student's t-test*) (Fig. 1C).

In addition, we investigated the effect of *ADAR1* loss-of-function over lncRNAs expression levels in a second breast cancer cell line, MDA-MB-436. Controls for ADAR1 mRNA expression, protein levels, and function downregulation were performed (Supplementary Fig. 4). In

the ADAR1 KD condition, we observed the expected upregulation of LINC00944 ($p = 0.0029$, *Student's t-test*) and APCDD1L-AS1 ($p = 0.0004$, *Student's t-test*). Nevertheless, no significant changes were found in LINC01003 ($p = 0.5977$, *Student's t-test*), and H1FX-AS1 presented a consistent upregulation even after *ADAR1 knockdown* ($p = 0.0031$, *Student's t-test*) (Fig. 1D).

3.3. ADAR1 and LINC00944 are interacting

Since LINC00944 expression levels were responsive to *ADAR1* gain- and loss-of-function, we decided to examine their potential interaction. To this end, we performed an ADAR1 RNA-immunoprecipitation followed by an RT-qPCR (RIP-qPCR). β-actin (*ACTB* gene) was used as a negative control since there is no reported interaction with ADAR1. Remarkably, we were able to recover ~30% of LINC00944 RNA in the ADAR1 immunoprecipitated fraction compared to the input sample, confirming that ADAR1 and LINC00944 are interacting in the breast cancer cell line MDA-MB-231 (Fig. 1E).

The canonical function of ADAR1 is the catalysis of the deamination reaction of adenosine to inosine in RNAs (A-to-I editing); thus, we next hypothesized that LINC00944 might be an editing target of ADAR1. To test this possibility, we searched for A-to-I variants in LINC00944 RNA

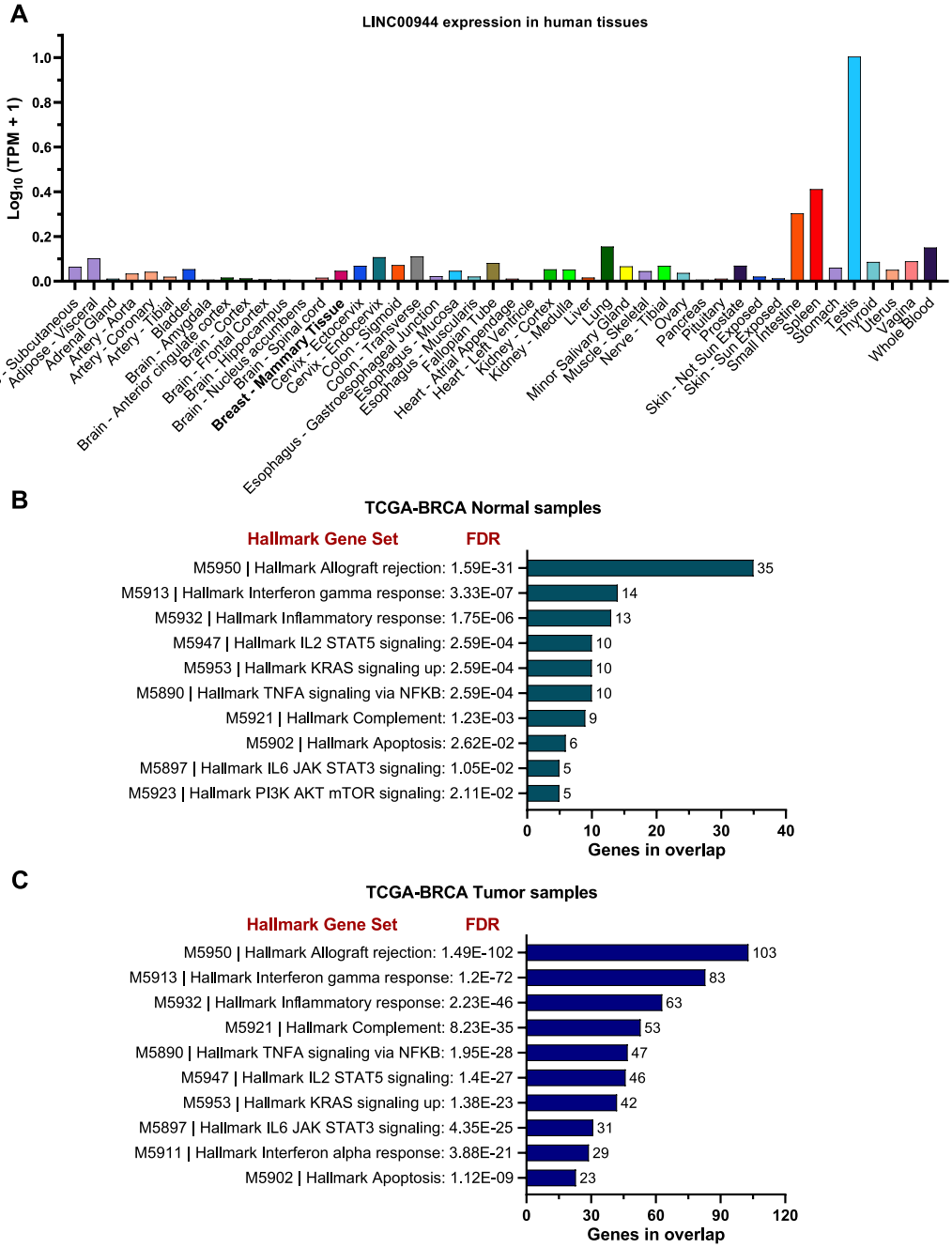

Fig. 2. Guilt-by-Association analysis connects LINC00944 to immune signaling pathways. (A) LINC00944 tissue-specific median expression (Log_{10} TPM+1) across 46 human tissues. Data was obtained from Genotype-Tissue Expression (GTEx) Project Portal (https://www.gtexportal.org/home/gene/LINC00944). (B) Guilt-by-Association analysis of LINC00944 co-expressed protein-coding genes followed by an overlap against the Hallmark Collection [37] in normal (solid tissue normal) and (C) tumor samples from the TCGA-BRCA cohort. Significant terms were ranked based on the number of genes in overlap and top 10 are depicted. TPM: Transcripts Per Million; FDR: False discovery Rate.

on the RNA-seq data originated from MDA-MB-231 cells overexpressing ADAR1 [30]. The bioinformatic pipeline for A-to-I variant detection showed no evidence of editing in LINC00944 RNA, suggesting that ADAR1 may alter LINC00944 expression levels by means of non-canonical functions.

3.4. LINC00944 has low expression in human tissues

To gain insight into LINC00944, we examined its expression across 47 human tissues based on data generated by the Genotype-Tissue Expression (GTEx) Project (https://gtexportal.org/home/). As shown in Fig. 2A, LINC00944 has a general low expression in normal tissues, as generally expected for lncRNAs [40]. The highest expression was found in Testis (Median TPM: 9.1, n=361), followed by Spleen (Median TPM: 1.6, n=241) and Small intestine (Median TPM: 1.0, n=187), while Breast showed a moderate expression (Median TPM: 0.11, n=459). The lowest expression values were found in brain and heart tissues (Heart-Left ventricle, Median TPM: 0.013, n= 432, and Brain-Nucleus accumbens, Median TPM: 0.013, n=246). Interestingly, no expression (Median TPM: 0) was reported in several brain tissues, i.e., the cerebellar hemisphere, cerebellum, caudate (basal ganglia), hypothalamus, putamen (basal ganglia), and substantia nigra.

3.5. LINC00944 is connected to immune signaling pathways in normal and tumor samples

To understand the LINC00944 function, we used the Guilt-by-Association method. To this end, we performed correlation analysis between LINC00944 and all protein-coding genes in normal and tumor datasets from the TCGA-BRCA cohort (*Pearson correlation*, $|r| \geq 0.3$ and FDR ≤ 0.01), followed by an overlap against the Hallmark Collection [37]. The results connected LINC00944 to immune system-related functions, as "Interferon-gamma response", "Inflammatory response", "IL2 STAT5 signaling" and "TNFA signaling via NFKB" gene set collections were significantly represented in normal and tumor datasets (Fig. 2B and C, and Supplementary Tables 2 and 3). To provide further support to this scenario, we took additional TCGA datasets in which LINC00944 was shown to have high expression, as Testis, Lung (Median TPM: 0.43, n=578), and Colon (Median TPM: 0.29, n=406) (Fig. 2A), and performed the Guilt-by-Association analysis. Remarkably, this analysis showed that in normal tissue from the lung, and in the Lung adenocarcinoma (TCGA-LUAD), Testicular Germ Cell Tumors (TCGA-

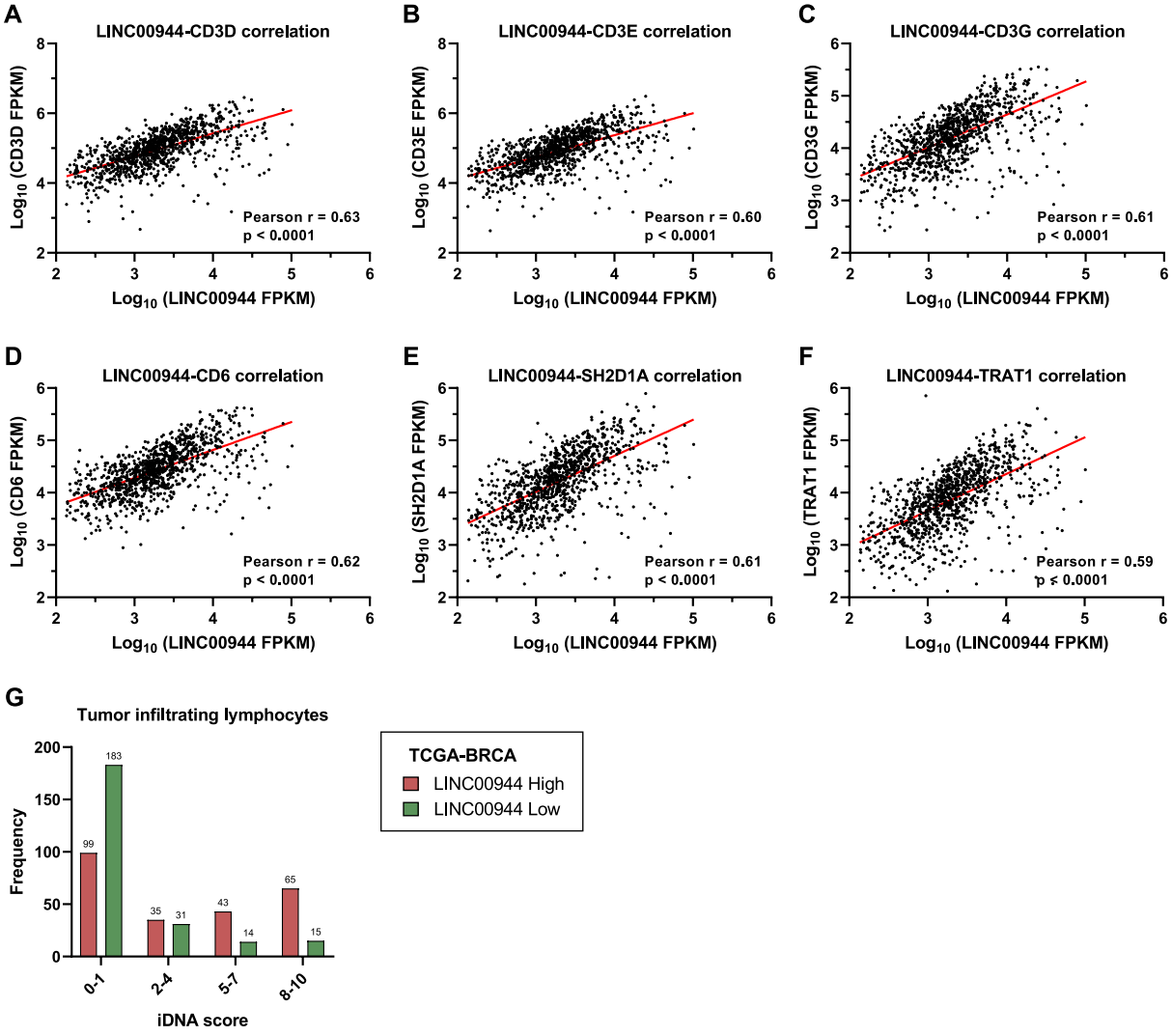

Fig. 3. LINC00944 expression positively correlates to tumor-infiltrating lymphocytes confident markers in breast cancer. Pearson correlation between LINC00944 and (A) CD3D, (B) CD3E, (C) CD3G, (D) CD6, (E) SH2D1A and (F) TRAT1 mRNA expression in TCGA-BRCA tumor samples. A p-value ≤ 0.05 was considered statistically significant. (G) iDNA score as an indicative of tumor-infiltrating T lymphocytes in patients expressing high (red) and low (green) levels of LINC00944 in TCGA-BRCA tumor samples. FPKM: Fragment Per Kilobase Million. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TGCT), and the Colon Adenocarcinoma (TCGA-COAD) cohorts, LINC00944 is also significantly related to immune functions (Supplementary Fig. 5A, B, C, and D, respectively). Thus, our results implied that LINC00944 appears to be participating in immune-related signaling pathways.

3.6. LINC00944 expression serves as a marker for tumor-infiltrating lymphocytes in breast cancer

Since the Guilt-by-Association analysis showed that LINC00944 appears to be participating in immune signaling pathways, we next sought to ascertain whether LINC00944 could be a useful marker for tumor-infiltrating lymphocytes (TILs). Two different approaches were employed for this purpose. In the first, RNA-seq data was used to assess the expression of high confidence marker genes for T-cell population [41] in tumor samples from TCGA-BRCA. As is shown in Fig. 3A–F, LINC00944 expression levels was positively correlated to the six confidence marker genes, CD3D ($p < 0.0001$, *Pearson* $r = 0.63$), CD3E ($p < 0.0001$, *Pearson* $r = 0.60$), CD3G ($p < 0.0001$, *Pearson* $r = 0.61$), CD6 ($p < 0.0001$, *Pearson* $r = 0.62$), SH2D1A ($p < 0.0001$, *Pearson* $r = 0.61$) and TRAT1 ($p < 0.0001$, *Pearson* $r = 0.59$), respectively, suggesting that LINC00944 could serve as marker for the presence of T-cells in tumor microenvironment. To provide additional support to this inference, we performed the same correlation analysis in the TCGA-TGCT, TCGA-LUAD, and TCGA-COAD datasets, finding significant and strong expression correlations in each case (Supplementary Table 4).

In the second approach, we reviewed previously published data in which exome reads mapping was employed to detect tumor-infiltrating T lymphocytes (immune DNA signature, *iDNA score*) in tumor samples from the TCGA-BRCA cohort [42]. Using this data, we observed that breast cancer patients expressing low levels of LINC00944 have lower iDNA scores, thus suggesting a lower infiltration of T lymphocytes in the tumor of those patients (Fig. 3G).

Together, our results suggest that LINC00944 expression positively correlates to TILs, indicating that LINC00944 could serve as a marker for tumor-infiltrating T lymphocytes in the tumor microenvironment.

3.7. Pro-apoptotic markers are downregulated in breast cancer patients expressing low levels of LINC00944

One major role of the immune system in tumor progression is to drive apoptosis in cancer cells [43]. With that in mind, we then aimed to elucidate whether LINC00944 expression correlated to alterations in programmed cell death. By interrogating the gene expression of apoptotic markers in TCGA-BRCA, we observed that patients expressing low levels of LINC00944 (LINC00944 low group) have a significant downregulation of the pro-apoptotic markers Bak (*BAK1*) ($p < 0.0001$, *Mann-Whitney test*) and Bax (*BAX*) ($p < 0.0001$, *Mann-Whitney test*) (Fig. 4A), and an upregulation of the anti-apoptotic markers Bcl2 (*BCL2*) ($p < 0.0001$, *Mann-Whitney test*) and $BclX_L$ (*BCL2L1*) ($p < 0.0001$, *Mann-Whitney test*) when compared to patients expressing high levels of LINC00944 (Fig. 4B). In addition, the initiator caspase, caspase-8 (*CASP8*) ($p < 0.0001$, *Mann-Whitney test*) and the executioner caspase, caspase-3 (*CASP3*) ($p = 0.0019$, *Mann-Whitney test*), were significantly downregulated in LINC00944 low group (Fig. 4C). The same tendency was observed when we analyzed the protein levels of the mentioned apoptotic markers (Supplementary Fig. 6). Taken together with these results, we suggest that low expression of LINC00944 in breast cancer patients is correlated to a decrease in the apoptotic program of tumor cells.

3.8. LINC00944 expression in breast cancer patients is correlated to clinicopathological parameters

To explore whether LINC00944 expression has a clinicopathological correlate in breast cancer, we examined the clinical data provided by

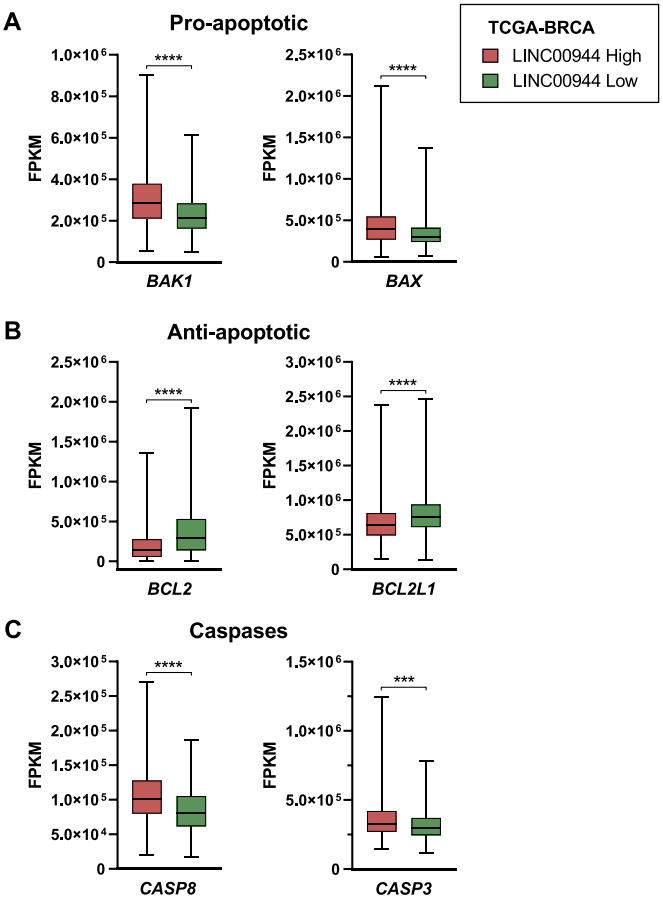

Fig. 4. Pro-apoptotic markers are downregulated in breast cancer patients expressing low levels of LINC00944. Expression levels of the (A) pro-apoptotic markers *BAK1* and *BAX*, the (B) anti-apoptotic markers *BCL2* and *BCL2L1*, and the (C) caspases 8 and 3 (*CASP8*, *CASP3*) in the TCGA-BRCA LINC00944 high (red) and low (green) groups. Data are depicted as Log_2(FPKM) and error bars represent Min to Max values. Data was analyzed using the *Mann-Whitney test*. **** $p < 0.0001$. ** $p = 0.0019$. A *p-value* ≤ 0.05 was considered statistically significant. $N = 250$. FPKM: Fragment Per Kilobase Million. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TCGA-BRCA, which revealed that LINC00944 expression was correlated to age at diagnosis ($p = 0.008$, *Fisher's exact test*), tumor size (AJCC Pathologic T; $p = 0.041$, *Fisher's exact test*) (Table 1), estrogen receptor status ($p = 1.84E^{-17}$, *Fisher's exact test*), progesterone receptor status ($p = 5.69E^{-09}$, *Fisher's exact test*) and breast cancer subtype by IHC ($p = 2.92E^{-09}$, *Chi-squared test*) (Table 2). However, no significant correlations were observed between LINC00944 expression and other clinicopathological parameters such as pathologic stages ($p = 0.329$, *Fisher's exact test*), metastasis to lymph nodes (AJCC Pathologic N; $p = 0.149$, *Fisher's exact test*), distant metastases (AJCC Pathologic M; $p = 0.441$, *Fisher's exact test*) and HER2 status ($p = 0.111$, *Fisher's exact test*).

3.9. LINC00944 low expression in breast cancer patients is correlated to poorer survival outcomes

Considering that alterations in immune pathways, lower lymphocytic infiltration, and dysregulation in cell death are key traits for tumor progression [43,44], we evaluated whether measuring LINC00944 expression had a clinical value assisting diagnosis of breast cancer. By interrogating RNA-seq expression data from TCGA-BRCA, we detected no significant differences between normal (n=113) and primary tumor (n=1045) samples ($p = 0.2014$, *Mann-Whitney test*) (Fig. 5A).

Table 1
Correlation between LINC00944 expression and clinicopathological parameters in breast cancer patients.

Parameters	n	LINC00944 expression		*p*-value
		High (*n*=250)	Low (*n*=250)	
LINC00944 expression				
FPKM (mean)		12,498	473.5	
Age at diagnosis				
≥50 years	363	168 (46.3%)	195 (53.7%)	0.008*
<50 years	132	79 (59.8%)	53 (40.2%)	
Pathologic Stage (AJCC Pathologic Stage)				
Stages I + II	378	194 (51.3%)	184 (48.7%)	0.329
Stages III + IV	110	50 (45.5%)	60 (54.5%)	
Tumor size (AJCC Pathologic T)				
T1 + T2	415	217 (52.3%)	198 (47.7%)	0.041*
T3 + T4	83	33 (39.8%)	50 (60.2%)	
Lymph node metastasis (AJCC Pathologic N)				
N0	249	134 (53.8%)	115 (46.2%)	0.149
N1 + N2 + N3	242	114 (47.1%)	128 (52.9%)	
Distant metastasis (AJCC Pathologic M)				
M0	413	200 (48.4%)	213 (51.6%)	0.441
M1	11	4 (36.4%)	7 (63.6%)	

TCGA-BRCA LINC00944 high and low expression groups were determined based on the LINC00944 *z*-score. Data were analyzed using the *Fisher's exact test*. A *p*-value ≤ 0.05 was considered statistically significant (*). AJCC: American Joint Committee on Cancer.

Table 2
Correlation between LINC00944 expression and receptor status in breast cancer patients.

Parameters	n	LINC00944 expression		*p*-value
		High (n=250)	Low (n=250)	
Estrogen receptor status				
Positive	331	125 (37.8%)	206 (62.2%)	$1.84E^{-17}$*
Negative	143	114 (79.7%)	29 (20.3%)	
Progesterone receptor status				
Positive	278	108 (38.8%)	170 (61.2%)	$5.69E^{-09}$*
Negative	195	129 (66.2%)	66 (33.8%)	
HER2 status				
Positive	73	30 (41.1%)	43 (58.9%)	0.111
Negative	254	133 (52.4%)	121 (47.6%)	
Subtype by IHC				
HR+/HER2-	181	73 (41.3%)	108 (59.7%)	$2.92E^{-09}$*#
HER2+	73	30 (41.1%)	43 (58.9%)	
Triple-negative	70	58 (82.9%)	12 (17.1%)	

TCGA-BRCA LINC00944 high and low expression groups were determined based on the LINC00944 z-score. Data was analyzed using the *Fisher's exact test*. # Data were analyzed using the *Chi-squared test*. A *p-value* ≤ 0.05 was considered statistically significant (*). HR: Hormone receptor. IHC: Immunohistochemistry.

Nevertheless, when we subdivided tumor samples by receptor status, we found that triple-negative breast cancer patients' samples (TNBC, *n*=113) had a significant upregulation of LINC00944 when compared to normal samples ($p < 0.0001$, *Kruskal-Wallis test* followed by *Dunn's multiple comparison test*) (Fig. 5B). This data suggests that LINC00944 high levels would be useful as a biomarker in TNBC patients.

Following, we wanted to further evaluate whether LINC00944 expression has a prognostic significance in breast cancer patients. For this purpose, we assessed two survival parameters, (1) the length of time from either the date of diagnosis or the start of treatment for that diagnosed patients are still alive, known as Overall Survival (OS), and (2) the length of time after primary treatment ends that the patients survive without any signs or symptoms of that cancer, known as Relapse-Free Survival (RFS) (*NIH-NCI Dictionary of Cancer Terms*). By using the web-based Kaplan Meier-Plotter (https://kmplot.com/analysis/) [32], we assessed survival on the breast cancer dataset, finding that both OS ($p = 0.011$, *Log-rank test*) and RFS ($p = 2.1E^{-06}$, *Log-rank test*) are significantly poorer in patients expressing low levels of LINC00944 (Fig. 5C and D, respectively). The analysis showed that the low expression cohort had a reduction of 40.8 months in OS (upper quartile survival 106.8 vs. 66 months), while a decrease of 22.4 months in RFS (upper quartile survival 53.6 vs. 31.2 months). Since LINC00944 expression was found significantly higher in TNBC patients, we examined the relapse-free survival in the TNBC cohort, finding a significant reduction in the LINC00944 low expression cohort (upper quartile survival 36.96 vs. 19.4 months) ($p = 0.022$, *Log-rank test*) (Fig. 5E). From this data, we can conclude that low expression of LINC00944 in these breast cancer patients is correlated to survival parameters.

4. Discussion

The ADAR1 upregulation observed in several cancer types has been largely described as an oncogenic feature [11]. Evidence has shown that ADAR1 has no preference for a specific type of RNA, being extensively described for interactions with mRNAs, microRNAs, and viral RNAs [2]. Despite the sharp rise in lncRNAs studies, only a few have addressed ADAR1-lncRNAs interactions. In this paper, we provided a transcriptomic analysis of the effect of ADAR1 over lncRNAs expression. Forty-one lncRNAs were differentially expressed (DE) after ADAR1 upregulation (*p*-adj ≤ 0.05), and we could further confirm that the *ADAR1* loss-of-function reverts the effect on expression in some of those in the breast cancer cell lines MDA-MB-231 and MDA-MB-436. A quick exploration of DE lncRNAs in PubMed (https://www.ncbi.nlm.nih.gov/pubmed) [45] showed that several lncRNAs were already linked to cancer development. For instance, APCDD1L-AS1 was found to have an important prognostic value in lung squamous cell carcinoma. Functional enrichment analysis of co-expressed genes revealed that critical pathways in cancer development as "positive regulation of cell migration" and "proteinaceous extracellular matrix" were enriched [46]. In our analysis, we showed that ADAR1 gain- and loss-of-function were capable of modulating the expression of APCDD1L-AS1, indicating that this lncRNA may also have a role in the breast cancer malignancy related to ADAR1. Another good example is the upregulation of the lncRNA FAM201A, which has been found to mediate metastasis of lung squamous cell cancer [47] and resistance to radiotherapy in non-small cell lung cancer [48] and esophageal squamous cell cancer [49]. In the ADAR1 OE condition, we found a significant upregulation of FAM201A, suggesting that this lncRNA may also have a role in the ADAR1-mediated tumorigenesis in breast cancer. On the other hand, the majority of DE lncRNAs have no associated literature, so we postulate them as principal candidates for exploring their function in cancer.

In the present study, we showed that LINC00944 expression levels were susceptible to ADAR1 up- and downregulation in two breast cancer cell lines, and the RNA-immunoprecipitation approach suggested that ADAR1 and LINC00944 may be interacting in breast cancer cells. Mechanisms by which ADAR1 can disrupt RNA expression levels through A-to-I editing range from stability impairment to alteration in splicing processing and nuclear retention in paraspeckles, among others [2]. In LINC00944, we found no evidence of A-to-I editing, excluding it as an underlying mechanism. On the other hand, it has been shown that ADAR1 can modify RNA expression levels by an interplay with other RNA-binding proteins, such as Dicer [24] and by and Staufen [50] and HuR [51]. These mechanisms are worth exploring in future studies for a complete understanding of the ADAR1-LINC00944 interplay.

LINC00944 has been outlined in cancer in previous publications. For

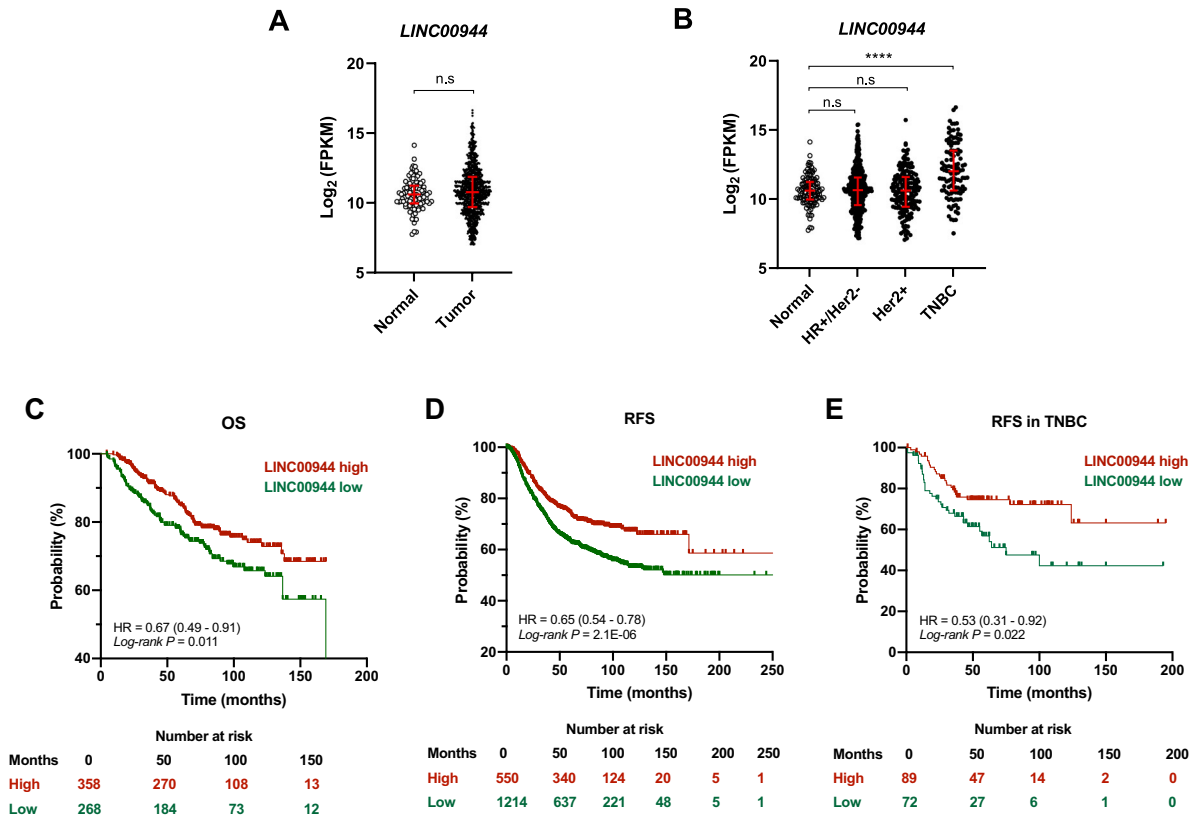

Fig. 5. LINC00944 low expression in breast cancer patients is correlated to poorer overall survival and relapse-free survival. (A) LINC00944 expression from the TCGA-BRCA cohort in solid tissue normal (n=113) and primary tumor (n=1045) samples. Data were analyzed using the *Mann-Whitney test*. (B) LINC00944 expression in normal (n=113), hormone receptor positive (HR+/HER2-), HER2 positive (HER2+) and TNBC samples. Data was analyzed using the *Kruskal-Wallis test* followed by *Dunn's multiple comparison test*. **** $p < 0.0001$. Expression data are depicted as Log_2 (FPKM) and error bars represent the median with interquartile range. (C) Kaplan-Meier survival curves and Risk tables for breast cancer patients based on LINC00944 expression. Overall survival curves in breast cancer patients ($n = 626$). (D) Relapse-free survival curves in breast cancer ($n = 1764$), and (E) TNBC ($n = 161$) patients. Red and green lines indicate high and low LINC00944 expression, respectively. *Log-rank test* was used to analyze data. A p-value ≤ 0.05 was considered statistically significant. '*ns*' indicates no significant difference between compared groups. TNBC: Triple-negative breast cancer; HR: Hazard Ratio. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

instance, LINC00944 expression has been shown as downregulated in colorectal cancer tissues from patients with liver metastasis [52] and reported as an epigenetically activated lncRNA in several cancer types when compared to their normal tissues [53]. Interestingly, LINC00944 was recently identified as an immune-related lncRNA in cancer, and associated to antimicrobials, cytokines, interleukins, antigen processing and presentation, natural killer cell cytotoxicity, TCR signaling, cytokine, chemokine, and interleukins receptors pathways in several cancer types, including breast cancer [54]. This data agrees with our findings, in which we reported that LINC00944 has a strong relationship with and may play a role in immune signaling pathways. Nonetheless, the molecular mechanism underlying LINC00944 participation should be further investigated.

The immune system plays key roles in cancer initiation and development [43], and the infiltration of immune cells into tumor microenvironment is a major factor in cancer progression [55]. By using publicly available data generated from RNA-sequencing and Exome sequencing, we observed that LINC00944 positively correlates to tumor infiltrating T lymphocytes in the tumor microenvironment, postulating LINC00944 as a good marker. These findings become especially relevant when considering that TILs have been related to a favorable clinical outcome in several cancer types, including colorectal cancer [56], non-small cell lung cancer [57], and breast cancer [58].

The execution of an effective immune response in the tumor microenvironment could lead to increased programmed cell death in cancer cells [44]; thus, we further studied a correlation between LINC00944 expression and apoptosis. In the present study, we showed that patients expressing low levels of LINC00944 had downregulation of pro-apoptotic markers, as the gene expression of *BAX* and *BAK1*, and the caspases *CASP8* and *CASP3* are significantly downregulated in breast cancer. Accordingly, the expression of the anti-apoptotic *BCL2* and *BCL2L1* is upregulated. These data may indicate that cells expressing low levels of LINC00944 have a decrease in the apoptotic process; yet, functional assays are needed for more evidence.

LINC00944 expression between normal and tumor samples from breast cancer was not statistically different, but we found that LINC00944 was statistically upregulated in triple-negative breast cancer (TNBC) patients. Along the same line, assessment of LINC00944 expression and receptor status showed that LINC00944 expression was positively correlated to estrogen and progesterone receptors and these findings are, all relevant to medical concerns regarding therapies.

Regarding clinicopathological parameters, we found that LINC00944 expression is correlated to the age at diagnosis and tumor size. Interestingly, samples coming from larger tumors had low expression levels of LINC00944. Taken with the aforementioned results, this finding may imply that those tumors have a higher fraction of malignant cells compared to tumor-infiltrating T lymphocytes and that the apoptotic program may be decreased. Kaplan-Meier survival analysis supported this idea, as Overall survival and Relapse-free survival decreased significantly in the LINC00944 low expression cohort.

5. Conclusion

In summary, LINC00944 expression was responsive to variations in ADAR1 levels, which is an important player in oncogenic processes. LINC00944 may have a role in immune signaling pathways since a strong connection with the immune system was found in normal and tumor tissues. Our results suggest that LINC00944 may be used with prognostic value, as its expression was correlated to tumor-infiltrating T lymphocytes in the tumor microenvironment and a decrease in pro-apoptotic markers. Finally, low LINC00944 expression indicated poorer survival outcomes in breast cancer patients.

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CRediT authorship contribution statement

PRdS, MS, and RA conceived and designed the project. PRdS and FM performed experiments. PRdS and AB analyzed the data. PRdS, MS and RA interpreted the data. PRdS wrote the manuscript. OH, KM, MS and RA discussed data, reviewed, and edited the manuscript. All authors read and approved the final manuscript. MS and RA jointly supervised this project.

Declaration of competing interest

PRdS, AB, FM and RA were Pfizer Chile employees. The authors have no further financial or non-financial conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.118956.

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