




RESEARCH PAPER



A novel lncRNA derived from an ultraconserved region: *lnc-uc.147*, a potential biomarker in luminal A breast cancer

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ABSTRACT

The human genome contains 481 ultraconserved regions (UCRs), which are genomic stretches of over 200 base pairs conserved among human, rat, and mouse. The majority of these regions are transcriptionally active (T-UCRs), and several have been found to be differentially expressed in tumours. Some T-UCRs have been functionally characterized, but of those few have been associated to breast cancer (BC). Using TCGA data, we found 302 T-UCRs related to clinical features in BC: 43% were associated with molecular subtypes, 36% with oestrogen-receptor positivity, 17% with HER2 expression, 12% with stage, and 10% with overall survival. The expression levels of 12 T-UCRs were further analysed in a cohort of 82 Brazilian BC patients using RT-qPCR. We found that *uc.147* is high expressed in luminal A and B patients. For luminal A, a subtype usually associated with better prognosis, high *uc.147* expression was associated with a poor prognosis and suggested as an independent prognostic factor. The lncRNA from *uc.147* (*lnc-uc.147*) is located in the nucleus. Northern blotting results show that *uc.147* is a 2,8 kb monoexonic transcript, and its sequence was confirmed by RACE. The silencing of *uc.147* increases apoptosis, arrests cell cycle, and reduces cell viability and colony formation in BC cell lines. Additionally, we identified 19 proteins that interact with *lnc-uc.147* through mass spectrometry and demonstrated a high correlation of *lnc-uc.147* with the neighbour gene expression and miR-18 and miR-190b. This is the first study to analyse the expression of all T-UCRs in BC and to functionally assess the *lnc-uc.147*.

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Introduction

Breast Cancer (BC) is the most commonly diagnosed type of cancer and the leading cause of cancer death among women [1]. BC is a heterogeneous disease with a range of clinical and molecular characteristics [2]. Based on immunohistochemical analysis, BC is subdivided into four main groups: luminal A, luminal B, HER2 (Human Epidermal Growth Factor Receptor 2), and triple-negative [3,4]. This classification is essential for prognosis and treatment decisions. While luminal A and luminal B are sensitive to hormone therapy, HER2 positive patients can benefit from monoclonal antibody therapy [5]. Triple-negative remains the most challenging group to treat, but new targeted therapies are becoming available, as, for example, PARP inhibitors in BRCA mutated patients [6]. Besides that, BC can also be classified according to their gene expression profile. Here, BC can be stratified in five groups: luminal A, luminal B, HER2 enriched, basal, and normal-like [7,8]. More recently, two more groups have been added to the molecular classification: claudin-low [9] and molecular apocrine [10,11]. Despite the BC general classification system and the existing

guidelines for the stratification of patients inside each group, the wide range of diversity observed in each subtype demands additional molecular markers, in order to offer personalized therapy and to improve BC survival rates [12,13].

Potential novel candidates to be used for molecular characterization of BC are the ultraconserved regions (UCRs). In 2004, Bejerano and colleagues described UCRs as genome regions larger than 200 nucleotides that are fully conserved among human, mouse, and rat and highly conserved across other disparate taxa [14]. Most UCRs are known to be transcribed ultraconserved regions (T-UCRs), in both normal and malignant tissues [15], but most of their transcripts remain uncharacterized [16]. Recently, these regions have been demonstrated to participate in several cellular processes, such as differentiation [17–19], metabolism [20,21], response to hypoxia [22], drug resistance [15,23], and cancer development and progression [15,16,24,25]. In addition, the differential expression of T-UCRs was associated with different types of cancer [26–28] (Fabris and Calin, 2017; Rossi et al., 2008; Habic et al., 2019), including cervical cancer [29], colorectal cancer [15,30], hepatocellular carcinoma [31],

neuroblastoma [32], and others [15,25,33,34]. In BC luminal A patients, overexpression of uc.63 is associated with poor prognosis [35], and uc.38 is downregulated in BC patients [36]. Thus, these studies highlight the importance of T-UCRs in the cellular environment and, consequently, in cancer biology. Moreover, the above mentioned associations suggest that T-UCRs-expression profiles can be a useful tool to differentiate human cancer types and improve its diagnosis and prognosis.

In this study, we expand the knowledge of T-UCRs in breast cancer by screening the expression of all 481 T-UCRs in BC patients. We found that 63% of transcripts correlated with some clinical and/or molecular parameter of BC. In addition, we further investigated 12 T-UCRs in a cohort of Brazilian BC patients and confirmed that two of those transcripts are differentially regulated in BC patients. Here, we provide a comprehensive characterization of the biology and the role of lnc-uc.147 in BC development.

Material and methods

Bioinformatic analysis

The statistical analyses for the TCGA data were performed in R (version 3.4.1) (<http://www.r-project.org/>), and the statistical significance was defined as a p-value less than 0.05. The sequences of 481 ultraconserved elements (ucr) in the human genome, their genomic coordinates in GRCh34/hg16, as well as their type (exonic, nonexonic), and the closest gene were acquired from <https://users.soe.ucsc.edu/~jill/ultra.html> (Bejerano *et al.*, 2004). We used UCSC Genome Browser LiftOver to lift the coordinates from GRCh34/hg16 to GRCh37/hg19 assembly. The expression of the ultraconserved regions, quantified as RPKM (reads per kilobase of transcript per million mapped reads) in primary tumours, were retrieved from TANRIC (https://ibl.mdanderson.org/tanric/_design/basic/main.html) [37]. We downloaded clinical information for the TCGA patients with invasive breast carcinoma (BRCA) from cBioPortal (<http://www.cbioportal.org/>) (cohort: TCGA provisional). The PAM50 subtype of the samples was acquired from [38]. We ended up with 827 cases with uc expression and clinical information. From Genomic Data Commons Data Portal, we downloaded FPKM (fragments per kilobase of transcript per million mapped reads) files in order to retrieve gene expression quantification data for the host genes of the ultraconserved regions in same tumours. The log₂-transformation was applied to the mRNA data.

The relationship between overall survival and covariates (mRNA expression levels and clinical parameters age and stage) was examined using a Cox proportional hazard model. A multivariate Cox proportional hazard model was fitted, including the clinical parameters and mRNA expression significance in the univariate analysis. These analyses were conducted for all the breast cancer cohort and also separately on each subtype as defined by PAM50 or immunohistochemistry. To visualize the association between genes that identified significant prognostic factors and survival, we first used the log-rank test to find the point (cut-off) with the most significant (lowest p-value) split in high and low mRNA

expression groups. Then, we used the Kaplan–Meier method to generate plots for this cut-off.

The t-test or analysis of variance (depending on the number of groups considered) was applied to normally distributed data. Otherwise, the Mann–Whitney–Wilcoxon test or Kruskal–Wallis test was applied to assess the relationship between mRNA expression and PAM50 status. The normality of the data was prior tested with a Shapiro–Wilk test. The data are presented as box-and-whisker plots (box plot represents first: lower bound; and third, upper bound quartiles, whiskers represent 1.5 times the interquartile range). The Pearson correlation test was applied to measure the strength of the association between the expressions of T-UCR, host genes, and other genes of interest. TANRIC platform (https://ibl.mdanderson.org/tanric/_design/basic/main.html) [37] also was used to analyse the correlation between the somatic copy number and microRNA expression with lnc-uc.147 expression. MiRDB was used for miRNA target prediction (<http://mirdb.org/>).

Patients samples

16 luminal A, 36 luminal B, 10 HER2-enriched, and 40 triple-negative Brazilian BC samples were obtained from Hospital Nossa Senhora da Graças, Curitiba, Paraná, Brazil. Tissue samples were obtained from fresh surgical specimens frozen in RNA later and stored at -80°C . All the samples were obtained with the patients' informed consent, and the subtypes were immunohistochemically confirmed. The protocol for studying biological markers associated with disease outcome was approved by the medical ethics committee of the Universidade Federal do Paraná (19,870,319.3.0000.0102-CONEP).

Cell cultures and growth conditions

MDA-MB-231, MDA-MB-436, SK-BR-3, BT-474, MCF-7, T-47D, ZR-75-1, and CAMA-1 cells were cultured in Dulbecco's Modified Eagle's Medium DMEM (LONZA) supplemented with 10% FBS (Gibco) and Penicillin-Streptomycin (Gibco), 100 U/mL and 100 $\mu\text{g}/\text{mL}$ respectively. ZR-75-1 were cultured in RPMI medium supplemented with 10% FBS and 1% Penicillin-Streptomycin. The MCF10A were cultured in DMEM/F12 (LONZA, USA) supplemented with 2.5 mM of L-glutamine, 20 ng/ml of epidermal factor growth (EFG), 0.01 mg/ml of insulin, 500 ng/ml of hydrocortisone, 0.5% of Penicillin-Streptomycin and 5% of horse serum.

RNA isolation, cDNA synthesis, and RT-qPCR

RNA from cells and tissues were extracted using the Quick RNA Miniprep Kit (ZymoResearch, USA). All RNAs were digested by DNase (Ambion, USA). The quality and concentration of the RNA were assessed using the nanodrop ND-1000 instrument (NanoDrop Technologies, Thermo Scientific, USA) and Bioanalyser (Agilent, USA). cDNA was synthesized using the SuperScript III cDNA kit (Invitrogen), and diluted cDNA was used for RT-PCR analysis using iQ SYBR Green Supermix (Bio-Rad) with the appropriate primers (Supplemental Table S4). The $2^{-\Delta\Delta\text{Ct}}$ method was used to calculate the relative abundance of

RNA genes compared with two of the following genes GAPDH, actin-beta, TBP, and u6 expression.

Strand-specific RT was made in 20 μ L, where 100 ng of RNA was transcribed to strand-specific cDNA. Two reverse transcription reaction mix was made for strand-specific cDNA synthesis: one to detect uc.147 sense-oriented transcript (using a forward primer for uc.147 and reverse primer for actin beta) and other for uc.147 antisense-oriented transcript (using reverse primers both for uc.147 and actin beta). Of note, we used only actin beta reverse primer in both reaction mix because it can recognize actin beta's mRNA. Strand-specific cDNAs were diluted 5-times with nuclease-free water to be used in qPCR analysis (Supplemental Table S4).

RACE cloning and northern blotting

To identify the 5'- and 3'-end of the uc.147 transcript, BT474 cell total RNAs were treated with DNase I (RNase-free) (Invitrogen), and the SMARTer RACE cDNA Amplification Kit (Clontech) was used, according to the manufacturer's instructions. The cDNA ends were amplified with the Platinum Taq DNA Polymerase High Fidelity (Invitrogen), and gene-specific primers (listed in Supplemental) were used. We performed a nested PCR with the nested universal primer provided with the kit and the nested gene-specific primers listed in Supplemental Table S4. The PCR fragments were then run on a 1.5% agarose gel, and DNA was extracted with the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. The RACE products were cloned into a TOPO[®] TA pCR[®]2.1 cloning vector (Invitrogen) according to the manufacturer's instructions, and the inserts were sequenced using the M13 primers. For northern blotting, a total RNA was electrophoresed on 15% PAA-urea gels (Calin et al., 2002). RNA source was BT474 cell line with no treatment, and cells treated with uc.147 siRNA.

siRNA treatment

CAMA-1 and BT474 cells were seeded 1 day before onto six-well plates, reaching a total of 200,000 for 50–70% confluence. The cells were transfected with 100 nM siRNA scramble, siRNA uc.147.1, and siRNA uc.147.2, and 20 nM siRNA *LRBA*.1, and siRNA *LRBA*.2. (Supplemental Table S4). For the transfection, we used lipofectamine 2000 reagent (Invitrogen) for 24, 48, and 72 h. We obtained the best results within 24 h. All siRNAs were provided by Sigma Aldrich.

Subcellular fractionation

The separation of nuclear and cytosolic fractions was performed using the PARIS Kit (Life Technologies) according to the manufacturer's instructions in two different cell lines CAMA-1 and BT474. The detection level of U6 and GAPDH was used as a nuclear and cytosolic marker, respectively [39].

Cell viability

For cell viability assay, CAMA-1 and BT474 cells treated with siSCR, siuc.147, or si*LRBA* were plated in 96 well plates.

A total of 5000 cells were seeded in each well. The cell viability was read after 24, 48, 72, and 96 hours after transfection. At each day the media were changed to 100ul of media + MTS, incubated for 4 hours, and read at 580 nm.

Apoptosis

The apoptosis ratio was analysed using the Annexin V-FITC Apoptosis Detection Kit. At 48 h after transfection, cells were harvested and resuspended in binding buffer containing Annexin V-FITC and PI according to the manufacturer's instructions. The samples were analysed by flow cytometry (BD Biosciences, USA). Cells were discriminated into viable cells, necrotic cells, and apoptotic cells using BD FACS Vantage[™] cytofluorimeter (BD Biosciences, USA), and then the percentages of apoptotic cells from each group were compared.

Cell cycle

For cell-cycle analysis, the cells were harvested 24 h after treatment with siSCR, siuc.147 or si*LRBA*, fixed in 70% ethanol, and stained in a solution containing 10 μ g ml⁻¹ of propidium iodide (Sigma-Aldrich), 10,000 U ml⁻¹ of RNase (Sigma-Aldrich) and 0.01% of NP40 (Sigma-Aldrich). After 30–60 min, the samples were analysed by flow cytometry using a BD FACS Vantage[™] cytofluorimeter (BD Biosciences). Analysis of samples were carried out acquiring 10,000–15,000 events/sample on FACS Calibur (BD). ModFit software was used to analyse cell cycle phases.

Colony-forming assay

The cells were seeded in 6-well plate (500 cells per well) and allowed to grow for 20 days at 37 C in a 5% CO₂ humidified incubator after treatment with siRNA uc.147, siRNA *LRBA*, and siRNA scramble. The cells were fixed with 100% methanol at room temperature for 20 min, stained with 1% crystal violet at room temperature for 5 min, and washed with water until excess dye is removed. The number of colonies in each well was counted. Because of the low ability of BT474 to form colonies, we only have the results of CAMA-1. The experiment was performed in triplicate.

Pull-down assay

Lnc-uc.147 was first cloned into the TOPO TA vector following manufactory's instructions (TAKARA, USA). The pull-down protocol was adapted from [40]. The TOPO TA with lnc-uc.147 was cloned inside the pMS2 vector. The digestion of TOPO TA was done by enzymes Xba1 (1ul- 20,000 U/ul) and Spe1 (2ul - 10,000 U/ul). Also, Xba1 (1ul- 20,000 U/ul) was used to digest the pMS2 vector and generate complementary extremities. The pMS2 vector with lnc-uc.147 and an empty control vector were co-transfected with the pMS2-GST vector in HEK293 cells. After 48 hours, the supernatant was collected, and the protein concentration was determinate by the Bradford method.

The GST fusion proteins were immobilized in the GSH beads (GE healthcare; cat 17-0756-01). The beads were washed (3 times) with NT2 buffer. Reduced glutathione was

used to elute the complexes. The fractions were analysed in silver staining SDS-PAGE gel (Pierce™ Silver Stain for Mass Spectrometry). The obtained complexes were sent to the Proteomics and Metabolomics Facility core of MD Anderson Cancer Center to mass spectrometry analysis.

Western blotting

For immunoblotting analysis, proteins were quantified using Bradford assay (Bio-Rad, USA), with absorbance read at 595 nm on an ELISA reader (BioTek, USA), and the concentrations calculated using a standard curve. The proteins were not denatured for the anti-*LRBA*. Overall, 40 µg of proteins were loaded on 8% gel and transferred on a nitrocellulose membrane using a semi-dry Transfer System, according to the manufacturer's instructions. The membrane was stained with Ponceau S to make sure that equal amounts of proteins were loaded in each lane. The membranes were then incubated for 2 h at room temperature with TBST containing 5% non-fat dry milk. The membrane was probed overnight at 4 C with the primary antibody, then horseradish peroxidase-conjugated secondary antibody (Cell Signalling) was added at a dilution of 1:3000. The following primary antibodies were used: anti-*LRBA* rabbit monoclonal antibody (Bethyl Cat.# A304-478A) diluted in 1:1000; and anti-beta-actin rabbit monoclonal antibody (BioVision Cat.#3917-30 T) diluted in 1:3000. The used secondary antibody was the anti-rabbit HRP linked (Cell Signalling Cat.#: 7074S) diluted in 1:5000. The bound antibodies were detected by enhanced chemiluminescence and using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis of the data was performed by Student's t-test or ANOVA. P-values of ≤0.05 were considered statistically significant. All experiments were performed in triplicate.

Results

T-UCRs expression in breast cancer correlates with patients' clinical parameters and overall survival

In order to investigate the expression level of T-UCRs and their association with clinical parameters, we evaluated the expression of the 481 T-UCRs using TCGA (The Cancer Genome Atlas) data available from TANRIC website [37]. The expression levels were given as reads per kilobase of transcript per million mapped reads (RPKM). The TANRIC website provides p-values for each comparison of the expression level of the UCRs with PR and ER status, HER2 status, stage, therapy, overall survival, and PAM50 status.

A total of 302 (62.78%) T-UCRs were significantly associated with at least one BC clinical parameter. From those, 148 (49%) were overexpressed and 154 (51%) were downregulated when comparing expression levels between tumour and

normal adjacent tissue. Each T-UCR can be associated with more than one clinical parameter. Therefore, we found that 209 (43.45%) T-UCRs were associated with PAM50, 172 (35.76%) with ER+, 158 (32.85%) with PR+, 80 (16.63%) with HER2+, 60 (12.47%) with stage, 176 (36.59%) with therapy outcome, and 46 (9.56%) were associated with overall survival (Supplemental Table S1). To enrich for novel lncRNA T-UCRs, we excluded all T-UCR which overlapped with mature mRNA sequences of protein coding genes. This left us with 125 intronic and 84 intergenic T-UCRs. T-UCRs identified in the majority of the breast cancer samples (>80%) were selected in order to avoid rare transcripts, generating a list of 33 T-UCRs. Analysing the subgroups of PAM50 gene signature from these 33 T-UCRs, we observed that 12 T-UCRs (uc.84, uc.138, uc.147, uc.193, uc.268, uc.271, uc.311, uc.376, uc.378, uc.427, uc.456, uc.475) had greater difference ($p < 0.01$) in expression levels among all subtypes. Finally, the expression level of these 12 T-UCRs were evaluated in a Brazilian cohort of BCs (Supplemental Figure S1 and Supplemental Table S2). Uc.147 and uc.193 expression were confirmed associated with subtype classification (Figure 1).

Our analysis based on TCGA data demonstrate that uc.147 has higher expression in luminal A subtype, while uc.193 has higher expression in basal subtype (Figure 1(a,b)). The differential expression of uc.147 and uc.193 was further confirmed in a Brazilian BC cohort (Figure 1(c,d)). In that cohort, we found that uc.147 is expressed at a significantly higher level in luminal A and B patients, when compared to HER2+ and triple-negative subtypes (Figure 1(c)). Moreover, we confirmed that uc.193 is highly expressed in triple-negative tumours versus luminal subtypes (Figure 1(d)).

We used univariate and multivariate Cox regression analysis on the TCGA cohort to predict the effect of T-UCRs on the patients' overall survival. For luminal A, a subtype usually associated with better prognosis, high uc.147 expression was associated with a poor prognosis (Figure 1(e)) with the highest hazard ratio (HR = 7.19, IC 95%: 1.23–42.09, $P < 0.05$) (Figure 1(e)). Therefore, we suggest that uc.147 could be an independent prognostic factor. Moreover, we also show that uc.193 expression could be an independent prognostic marker of poor survival for all BC (HR = 1.75, IC 95%: 1.21–2.52, $P < 0.01$) (Table 1 and Figure 1(f)).

uc.147 is located in an intronic region of the *LRBA* gene (ENSG00000198589). Even though in our first analysis the uc.193 appears to be intronic, a more detailed analysis demonstrated that part of its sequence overlap the 3'UTR of the *SYNCRIP* gene (ENSG00000135316). Interestingly, both uc.147 and uc.193 expression are correlated with their host genes ($r = 0.6$ and 0.9 , respectively with $p < 0.001$) (Supplemental Figure S2). Despite of that, *LRBA* and *SYNCRIP* expression are not correlated with survival (Table 1 and Supplemental Figure S2), which indicates that the T-UCRs could be directly involved with BC.

Both uc.147 and uc.193 demonstrated promising prognostic value. As uc.193 have associated with the TNBC that we know to have worse prognosis, it was expected that higher levels of uc.193 was also associated with worse prognosis in BC patients. But when we looked specifically to the TNBC subtype, the expression levels of uc.193 is not

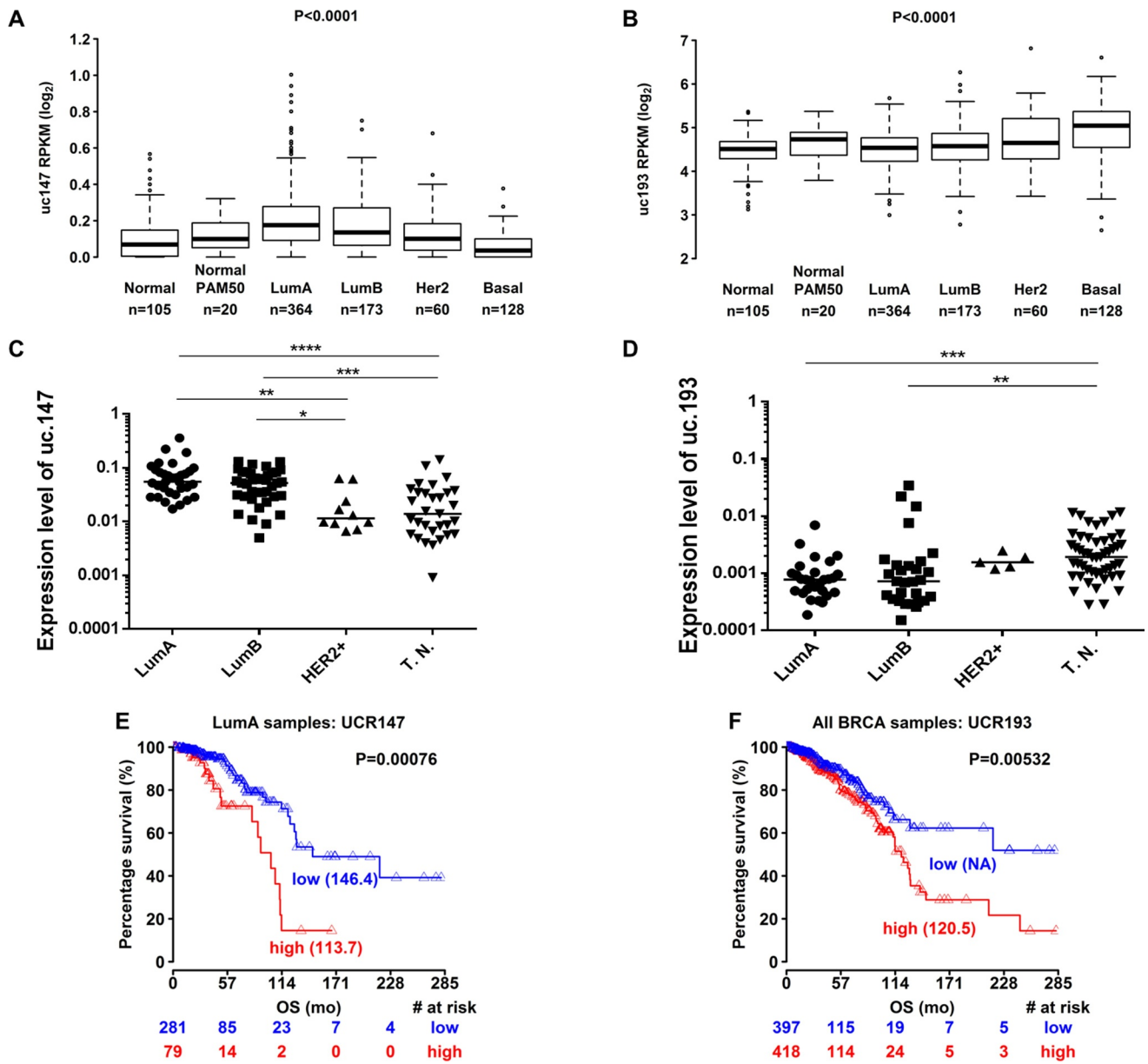


Figure 1. Expression analysis of uc.147 and uc.193. (a) and (b) Expression levels of T-UCRs (uc.147 in a and uc.193 in b) in all breast cancer subtypes from TCGA patients: Normal, Normal PAM50, Luminal a (LumA), Luminal b (LumB), HER2 overexpression (HER2+), and Basal. (c) and (d) Expression levels of T-UCRs (uc.147 in c and uc.193 in d) in the Brazilian BC cohort subtypes: Luminal A (LumA, n = 12), luminal b (LumB, n = 15), HER2+(HER2+, n = 5), and triple negative (T.N., n = 20). E. Overall survival for LumA BC patients according to uc.147 expression. f. Overall survival for BC patients according to uc.193 expression. The Kaplan–Meier method was used to generate the percentage of disease-free curves. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1. Summary of univariate and multivariate Cox regression analysis of overall survival and luminal A patients survival.

Variable	Univariate analysis				Multivariate analysis			
	HR	lower .95	upper .95	p-value	HR	lower .95	upper .95	p-value
BRCA								
Age (continuous)	1.03	1.02	1.05	1.92E-05	2.34	1.55	3.52	5.30E-05
Stage (III/IV vs. I/II)	2.34	1.55	3.52	5.30E-05	1.04	1.02	1.05	5.9E-07
uc.147	2.64	0.75	9.25	0.12971				
LRBA	1.01	0.99	1.04	0.25445				
uc.193	1.57	1.12	2.21	0.00962	1.75	1.21	2.52	0.0026
SYNCRIP	1.01	0.99	1.03	0.22748				
Luminal A								
Age (continuous)	1.03	1.01	1.05	0.01059	1.03	1.005	1.05	0.01752
Stage (III/IV vs. I/II)	1.75	0.89	3.45	0.10494				
uc.147	8.25	1.57	43.47	0.01276	7.19	1.23	42.09	0.028
LRBA	1.04	1.00	1.07	0.04808	1.03	0.99	1.07	0.123
uc.193	2.42	1.17	4.99	0.01692	2.31	1.1	4.85	0.02612
SYNCRIP	1.04	0.99	1.10	0.10606				

associated with a better or poor prognosis (Supplemental Figure S3). Although, uc.147 expression level is not associated with overall survival in BC patients, it is associated with overall survival inside the luminal A group (Supplemental figure S3 and Figure 1(e)). This suggests uc.147 expression levels as a potential additional prognostic marker for luminal A patients, which may be important for therapeutic decisions.

A novel long non-coding RNA resides within the 51st intron of *LRBA*

Taking into consideration that uc.147 expresses the highest levels in the luminal A sub-group among all PAM50 groups, and it also has the highest HR among the prognosis markers for the luminal A sub-group, we hypothesized that this

transcript should have an important oncogenic role in luminal A subtype. Thus, we focused our further analysis on uc.147.

uc.147 is an ultraconserved region of 308 bp located inside the 51st intron of the *LRBA* gene (Figure 2(a)). To initiate genomic characterization of lnc-uc.147, we first aimed to determine whether this T-UCR is transcribed in the same orientation as its host gene in BC cell lines. Total RNA was isolated and DNase treated from CAMA-1 (luminal A) and BT474 (luminal B) cell lines before we carried out two strand-specific RT-qPCR reactions, one with primer binding to the plus strand and one with primer binding to minus strand. We found that the predominant forms of lnc-uc.147 expressed in CAMA-1 and BT474 lineages is an antisense transcript in regard to *LRBA*, and the sense transcript was only poorly detectable (Figure 2(b)). Finally, we separated the

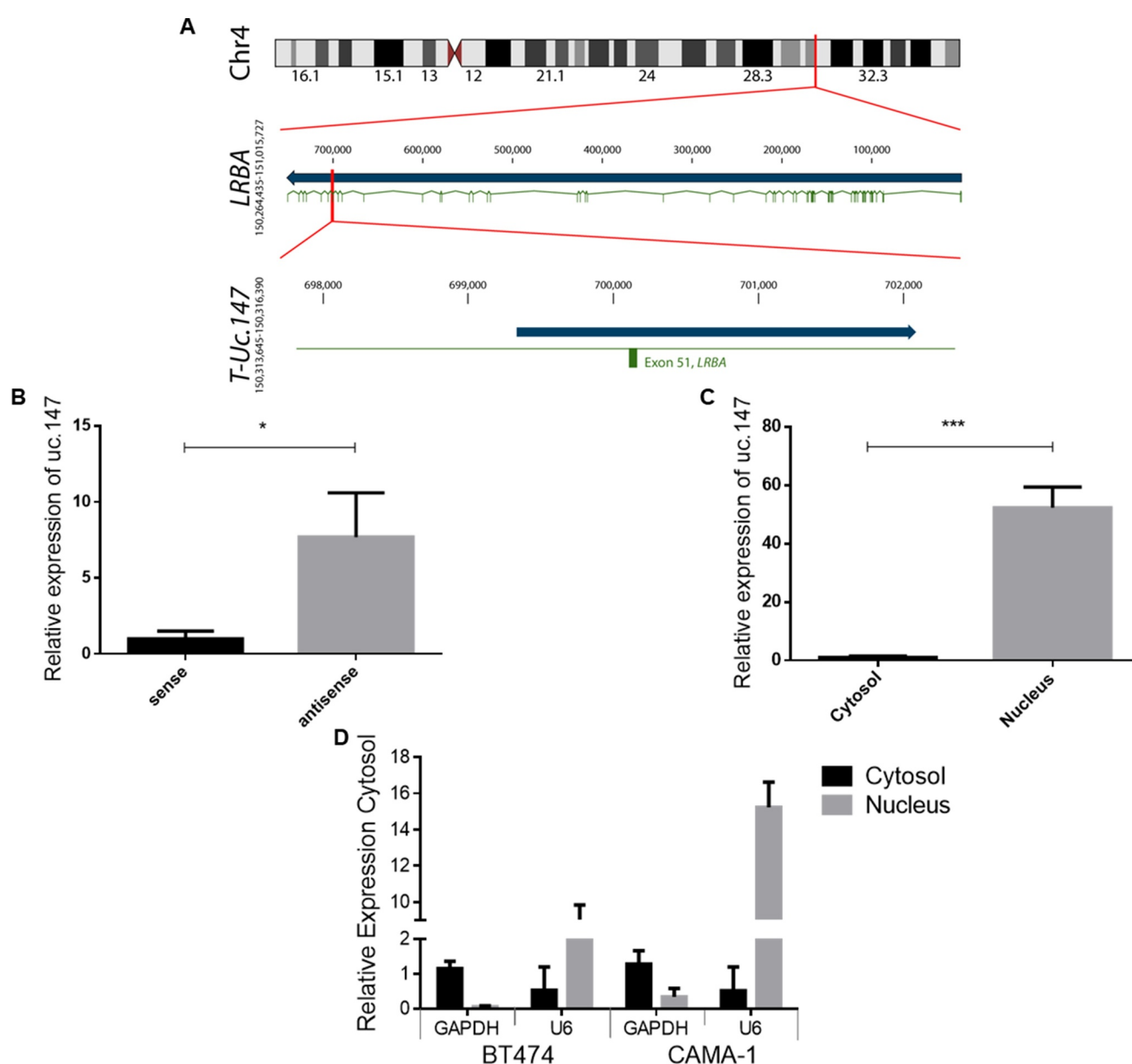


Figure 2. Characterization of lnc-uc.147. (a) Scheme of lnc-uc.147 transcript and locus in chromosome 4 (grey: *LRBA* gene/orange: uc.147 ultraconserved sequence and lnc-uc.147 transcript/ blue: *LRBA* exons). (b) Strand-specific RT-qPCR for lnc-uc.147. (c) RT-qPCR assays for detection of lnc-uc.147 performed on RNA extracted from nuclear and cytosolic fractions obtained from two different cell lines, CAMA-1 and BT474. (d) Detection level of GAPDH and U6 as cytosolic and nuclear markers, respectively. * $p < 0.05$. *** $p < 0.001$.

cytosolic and nuclear cell fractions and performed RT-qPCRs from each fraction to determine in which cell compartment the transcript is most abundant. The expression of *lnc-uc.147* was only detectable at the nucleus (Figure 2(c)). The GAPDH was used as a cytosolic control and the U6 as nucleus control (Figure 2(d)).

Northern blotting analysis were performed in BC cell lines (BT474 and CAMA-1) to determine the precise size and relative amount of *lnc-uc.147*, and it revealed that the transcript is approximately 3 kb long. To further define the exact size of *lnc-uc.147*, 3' and 5' RACE analysis were performed. For the cloning by RACE analysis, we first did a poly A + tailing in both RNA ends. From the 3' end we uncovered 1800 nt from the *lnc-uc.147* (Supplemental Figure S4). The sequence was colinear with the *LRBA* antisense sequence available at NCBI (National Center for Biotechnology Information). To completely uncover the 5' region of the *lnc-uc.147* transcript, we designed PCR primers based on the *LRBA* gene sequence corresponding to the region obtained from 3' RACE results. We performed a primer walking using the cDNA from *lnc-uc.147* as a reference. We then sequenced each amplified fragment and uncovered the final and complete sequence of the *lnc-uc.147* transcript – a mono-exonic RNA with 2746 nucleotides.

Knock-down of *uc.147* decrease cell viability and induces apoptosis in luminal breast cancer cells

Due to the high conservation of T-UCRs, we hypothesize that those transcripts have important cellular function in humans, as it is known that evolutionary conservation is a hallmark of many biological mechanisms. Using silico analysis, we were able to identify that the *lnc-uc.147* has 95% homology with Zebrafish sequences and 99% homology with sequences of other mammals. Further analysis on the sequence of this genomic region suggested that the evolution of this *locus* started within the first vertebrates (Supplemental Figure S5).

RT-qPCR was performed in nine BC cell lines to identify *lnc-uc.147* high-expressing cell lines (Figure 3(a)). To highlight the *lnc-uc.147* as a proper transcript and not only a passenger intronic expression, we performed an RT-qPCR in other intron of *LRBA* gene and compared the Ct values of both and with expressed exon of *LRBA* (Figure 3(b)). We could observe a major expression of the *lnc-uc.147* in comparison with a non-specific intron (Figure 3(b)). In addition, using TCGA data, we compared the expression levels of the *lnc-uc.147* with the expression levels of all introns from *LRBA* and all exons, and also observed a greater expression of *lnc-uc.147* in comparison with the all *LRBA* introns expression (Figure 3(c)).

To investigate the biological role of *lnc-uc.147* in BC, two luminal cell lines with high expressing levels of *lnc-uc.147* were chosen (CAMA and BT474). A siRNA-based approach, using two specific siRNAs targeting the *uc.147* ultraconserved sequence, was applied to investigate the phenotypic effects of *lnc-uc.147* knockdown (KD) (Figures 3 and 4). In BT474 cells, the siuc.147#1 reduces expression by 40%, and the siuc.147#2 reduces 60% (Figure 3(d)). On the other hand, in CAMA-1 cells, siRNA *uc.147.1* does not have any effect, but siRNA

uc.147.2 has the potential to inhibit 50% of *lnc-uc.147* expression (Figure 3(e)).

In BT474 cells, we saw a decrease in *LRBA* expression after siRNA treatment (Figure 3(d)). In addition, in CAMA-1 cells, the siRNA against *uc.147* has a weak effect on the *LRBA* expression (Figure 3(e)). Assays using a specific siRNA against the *LRBA* gene was performed and here only *LRBA* expression level was reduced (Figure 3(f,g)). Furthermore, silencing the *LRBA* gene leads to the complete knockdown of its protein, but the *LRBA* protein is still detectable when the siRNA against *uc.147* is used (Supplemental Figure S6).

After *lnc-uc.147* downregulation, BT474 and CAMA-1 cell viability decreased, but this effect was not observed after *LRBA* downregulation (Figure 4(a,b)). On BT474, both siRNAs against *lnc-uc.147* led to decreased cell viability (Figure 4(a)). Additionally, the knockdown of *lnc-uc.147* in CAMA-1 cells has an inhibitory effect in colony formation (Figure 4(c,d)). Colony formation was not assessed on BT474 due to its low ability to form colonies. In addition, we also performed the cell viability after the silence of *lnc-uc.147* in the MDA-MB-231, a cell line that did not express the *lnc-uc.147* and no effect was observed (Supplemental Figure S7).

Silencing of *lnc-uc.147* also leads to cell death in CAMA-1 (Figure 5(a)), but not in BT474 (Figure 5(b)). The cell death in CAMA-1 could be associated with an increase in G0/G1 cells and a reduction in G2/M events (Figure 5(a)). Although we observe a higher percentage of the G0/G1 phase in BT474, this difference was not significant (Figure 5(b)). These effects in the cell cycle, however, are only seen when *lnc-uc.147* is knocked down, but not when the *LRBA* host gene is knocked down (Figures 4 and 5). This suggests that *lnc-uc.147* and *LRBA* are two independent transcripts with different roles. Taken together, these findings show that *lnc-uc.147* plays an important role in the survival of BC cells.

Protein binding sites in *lnc-uc.147*

To understand the interactions of *lnc-uc.147* and to uncover its function we performed a pull-down assay [40]. We constructed two pMS2 vectors, one with the *lnc-uc.147* sequence and the other without, and then we co-transfected the constructed pMS2-GST vectors in HEK293 cells. The supernatant of each was collected separately and precipitated using GSH beads. After the precipitation, the proteins were added in SDS-PAGE gel and stained with silver. Two experiments were done independently. The gel was cut, and the proteins were identified using mass spectrometry.

The proteins identified in each gel were compared. First, we compared the two gels of the vector with the *lnc-uc.147* transcript and obtained a list of proteins. Second, we compared the proteins extracted from the gels of the empty vector. The protein lists were compared within each other, between gels, and we excluded the proteins that were common in both to finally obtain the proteins bound only to the *lnc-uc.147* (Table 2).

After the pull-down results, we did a literature search on the function of all proteins. We observed that most of them are related to cancerous cell processes, with emphasis in the cytoskeletal structure, centrossomal organization, and

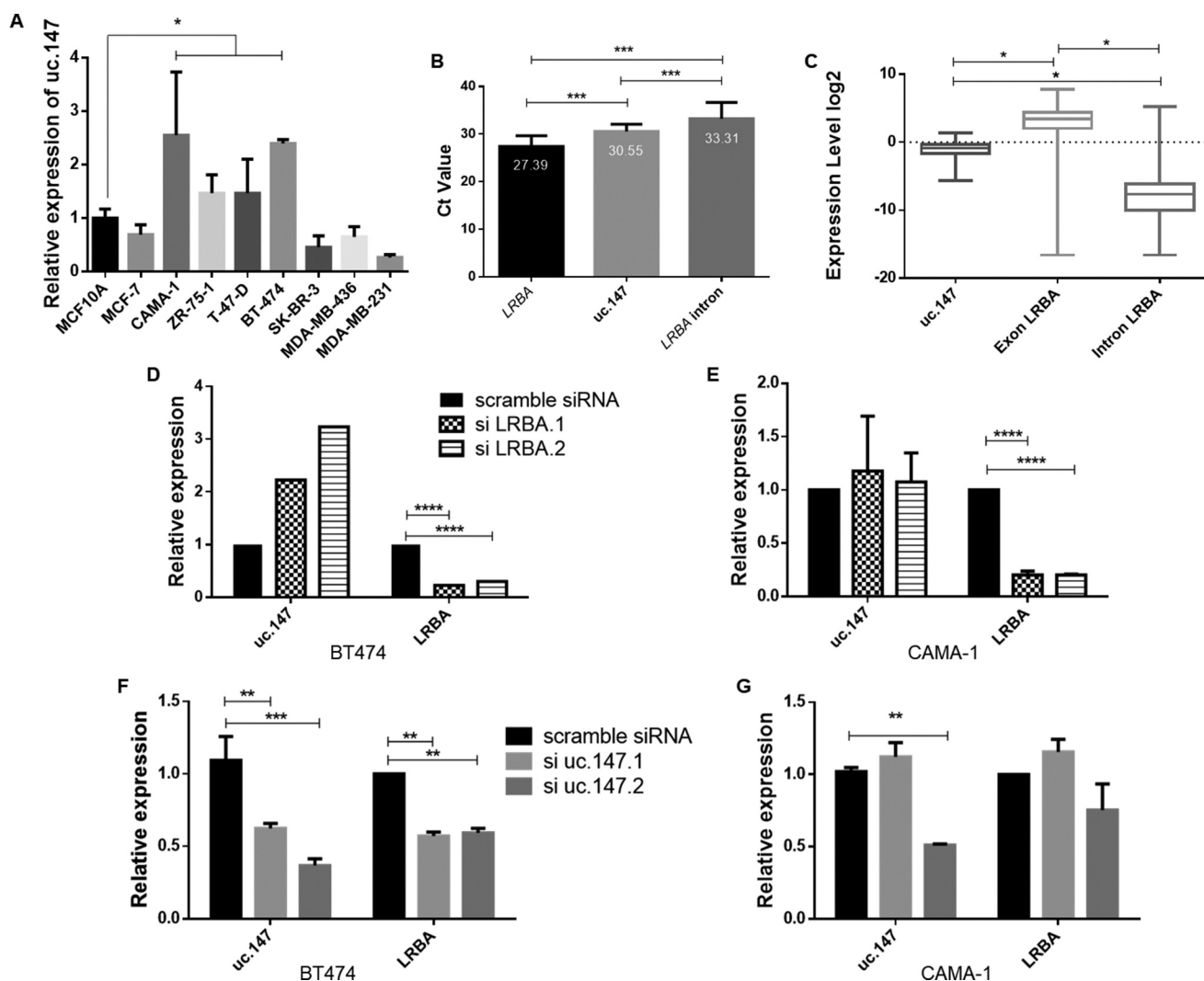


Figure 3. Lnc-uc.147 knockdown reduces cell viability, colony formation and increases apoptosis. (a) Expression levels of Lnc-uc.147 in different BC cell lines. (b) Expression levels of LRBA, uc.147 and a intron of LRBA in CAMA-1. The representative CT values are 27.39, 30.55 and 33.31 respectively. (c) Expression levels of uc.147, all LRBA exons and all LRBA introns retrieve from TANRIC website. (d) Relative expression of Lnc-uc.147 and LRBA after siRNA uc.147.1 and siRNA uc.147.2 (100 nM) treatment in BT474. (e) Relative expression of Lnc-uc.147 and LRBA after siRNA uc.147.1 and siRNA uc.147.2 (100 nM) treatment in CAMA-1. (f) Relative expression of LRBA and Lnc-uc.147 after siRNA LRBA.1 and siLRBA.2 (20 nM) treatment in BT474. (g) Relative expression of LRBA and Lnc-uc.147 after siRNA LRBA.1 and siLRBA.2 (20 nM) treatment in CAMA-1.

epithelial–mesenchymal transition (EMT). Some of these proteins have already been associated with BC: high expression of KRT17 was associated with poor survival and aggressiveness of several cancers including BC [41,42]; and CCT8, TEX10, TDRD3, and DKC1 overexpression were associated with BC progression [43–47]. CALML5 was found as a biomarker of BC [48].

PES1 contains a BC-associated gene 1 (BRCA1) C-terminal (BRCT) protein domain, and it is oestrogen inducible. The sensibility to oestrogen is interesting because Lnc-uc.147 was associated with oestrogen receptor-presence, and its upregulation was associated with survival only in Luminal A patients. In addition, PES1 expression gradually increases during BC development and progression [49,50]. Another protein that interact with the oestrogen receptor and could potentially have a correlation with Lnc-uc.147 is LRRC9, and its overexpression is associated with poor prognosis in BC [51,52].

Despite the fact that some of these proteins had already been associated with BC, no studies have reported any complexes among them. In addition, we analysed the overall survival ratio in BC of these proteins through the Oncolnc website (<http://www.oncolnc.org/>). Of 19 proteins, only two (KRT17 and LRRC59) are associated with overall survival in BC (data not shown).

To better understand the interaction among these proteins and Lnc-uc.147, we analysed protein interactions in tools such as String (<https://string-db.org/>) and Interactome (<http://www.interactome-atlas.org/>) (Supplemental Figure S8 and S9). In String, we searched for the interactions that had already been confirmed in other studies. We observed that some of these proteins interact with each other, forming networks such as the one including RHOA and CIT, and WDR18, TEX10, PES1, NOP53 (GLTSCR2), DKC1 and ZC3HAV1 (Supplemental Figure S8).

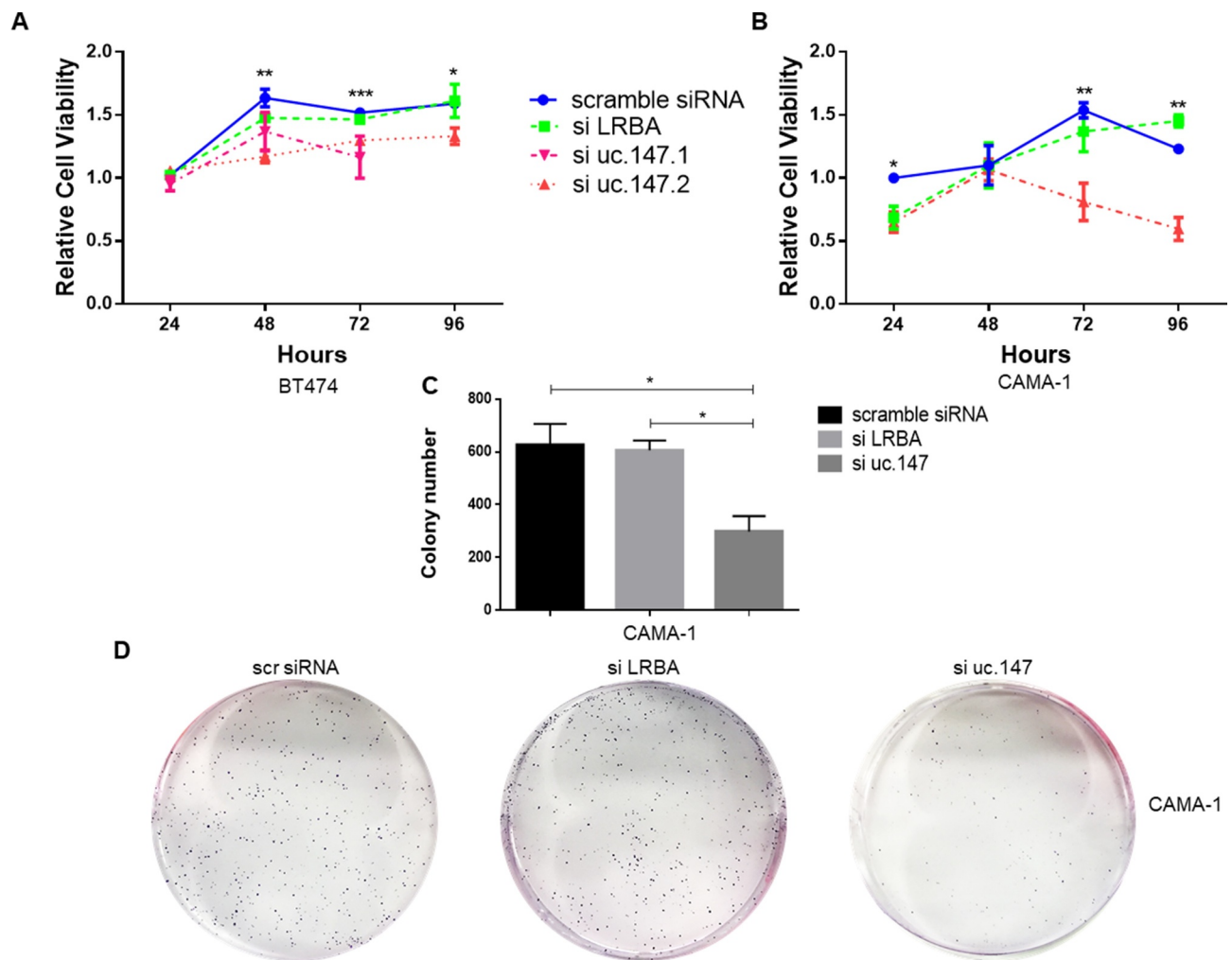


Figure 4. Cell viability and colony formation assays of lnc-uc.147. (a) and (b) Cell viability in BT474 (a) and CAMA-1 (b) after treatment with siRNA uc.147.1 (100 nM), uc.147.2 (100 nM), and siRNA *LRBA* (20 nM), at 24, 48, 72, and 96 hours. (c) Number of CAMA-1 colonies after siRNA treatment with uc.147.2 (100 nM) and siRNA *LRBA* (20 nM). (d) Representative plates of the colony formation.

Of the 19 proteins found in the pull-down followed by mass spectrometry, 17 of the genes encoding these proteins demonstrated a negative or positive correlation with the lnc-uc.147 (Supplemental Table S3), reinforcing a potential association among these molecules. However, more studies will be necessary to fully uncover the interactions of lnc-uc.147 and the network of proteins that this transcript interacts.

Correlation with neighbours lnc-uc.147 genes, somatic copy number and microRNAs

Aiming to identify potential cis-acting mechanisms, we analysed the correlation expression of lnc-uc.147 with the neighbour genes *MAB21L2*, *PITX2*, *TACR3*, *CXXC4*, *HNRPD*, *HNRPDL*, *CDKL2*, *G3BP2*, and *DCLK2*. Eight from nine genes analysed showed a significant correlation with lnc-uc.147 (Supplemental Table S3). A positive correlation was also found between lnc-uc.147 and two downstream UCRs uc.148 and uc.149 (Supplemental Table S3). It was analysing a correlation between the somatic copy number aberration in the *lnc-uc.147* locus and neighbours gene that we found

a positive correlation of this transcript with the host gene *LRBA* and its downstream gene *MAB21L2*.

In addition, looking for microRNAs and lnc-uc.147 expression, we found a negative correlation with mir-18a ($r = -0.31$, $P = 1.18 \times 10^{-12}$) and a positive correlation with mir-190b ($r = 0.30$, $P = 3.57 \times 10^{-12}$). We also predict the binding between lnc-uc.147 and miRNAs through the miRDB website [53]. A total of 125 miRNAs were predicted to bind to lnc-uc.147 (data not shown), we claimed attention for the mir-190 with a target score of 100% and correlated with this transcript.

Discussion

We analysed all 481 T-UCRs in TCGA data and found that most of them (~63%) are associate with some clinical parameters. In addition, uc.147 and uc.193 differential expression were evidenced in a Brazilian cohort. Also, the overexpression of uc.147 is associate with poor prognosis in luminal A patients and overexpression of uc.193 with both luminal A and BC poor survival. Previously, only two T-UCRS were investigated in BC, the overexpression of uc.63 is associated with poor prognosis in Luminal A patients [35], and

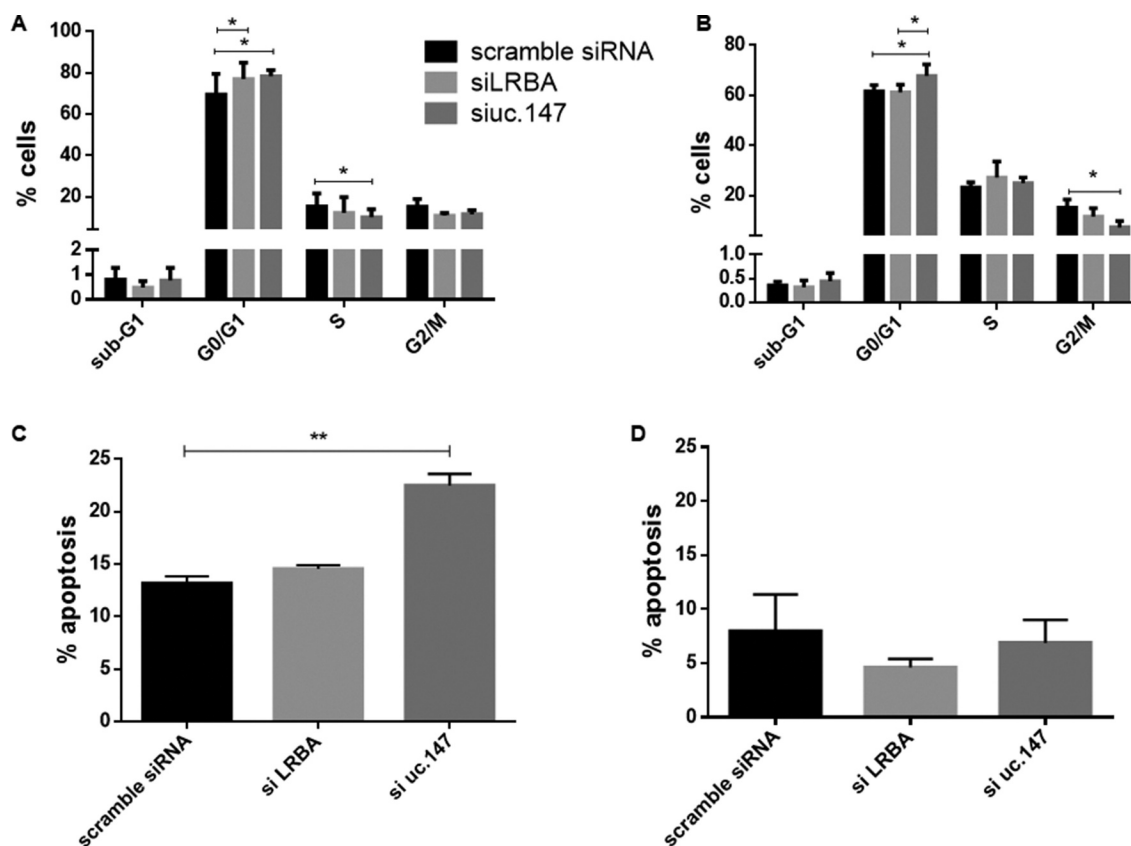


Figure 5. Cell cycle and apoptosis after silence of lnc-uc.147 (a) and (b) Cell cycle analysis in CAMA-1 (a) and BT474 (b) after siRNA uc.147.2 (100 nM) and siLRBA (20 nM). (c) and (d) Apoptosis ratio after CAMA-1 (c) and BT474 (d) treatment with siRNA uc.147.2 (100 nM) and siLRBA (20 nM). * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

Table 2. Results of pull-down assay indicating the coverage of proteins binding to the lnc-uc.147.

Protein	Protein ID	Official Symbol (local)	Coverage	Function
Nascent Polypeptide Associated Complex Subunit Alpha	ENSG00000196531	NACA (N)	40.85%	Epithelial–Mesenchymal Transition
Keratin 17	ENSG00000128422	KRT17 (+C)	26.16%	Cytoskeletal System
Dyskerin Pseudouridine Synthase 1	ENSG00000130826	DKC1 (+N)	21.4%	rRNA processing and TERC stabilization
ATP synthase subunit alpha	ENSG00000152234	ATP1 (+C)	19.48%	ATP production
Zinc finger antiviral 1	ENSG00000105939	ZC3HAV1 (N/C)	17.6%	mRNAs Stability
Calmodulin Like 5	ENSG00000178372	CALML5 (N/C)	16.44%	Epithelial–Mesenchymal Transition
Pescadillo Ribosomal Biogenesis factor 1	ENSG00000100029	PES1 (N/C)	14.25%	Contain a BRCA1 Domain
Scaffolding protein	ENSG00000083544	TDRD3 (+N)	13.85%	Transcription Activation
Spermatid Perinuclear RNA Binding Protein	ENSG00000165209	STRBP (+N)	11.25%	RNA binding
POM121 Transmembrane Nucleoporin Pseudogene	ENSG00000196313	LOC102725072 (+N)	8.66%	Transmembrane Nucleoporin
WD Repeat Domain 18	ENSG00000065268	WDR18 (N)	7.84%	DNA replication
Ras Homolog Family Member A	ENSG00000067560	RHOA (N/C)	7.77%	Cytoskeletal System
Glioma Tumour Suppressor Candidate Region Gene 2 Protein	ENSG00000105373	NOP53 (N/C)	4.60%	Chromosome Stability
Leucine Rich Repeat Containing 59	ENSG00000108829	LRRC59 (N)	3.91%	ER binding
Centrosomal Protein 68kDa	ENSG00000011523	CEP68 (C/N)	3.39%	Chromosome Stability
Chaperonin Containing TCP1 Subunit 8	ENSG00000156261	CCT8 (+C)	2.11%	Cytoskeletal System
Centrosomal protein 131	ENSG00000141577	CEP131 (+C)	2.1%	Cytoskeletal System
Testis Expressed 10	ENSG00000122966	TEX10 (N/C)	1.97%	Epithelial–Mesenchymal Transition
Citron Rho-Interacting Serine/Threonine Kinase	ENSG00000122966	CIT (N/C)	0.45%	Cytoskeletal System

The coverage indicates the quantite of bounding proteins. Local = subcellular localization. N = nucleus; C = cytosol; +N = most nuclear; +C = most cytosolic.

downregulation of uc.38 is associated with the worst outcome of BC patients [36].

Uc.147 is a non-characterized T-UCRs, and our study helps to better understand these transcripts lnc-uc.147 is antisense and independent from its host gene LRBA. This transcript has a 2746 nucleotides, and the sequence includes an intron and one exon of the LRBA gene. The independent transcription indicates that this T-UCRs is a long coding RNA and could participate in some cellular processes of cancer development.

As lnc-uc.147 was highly expressed in ER-positive subtypes and associated with poor survival in luminal A patients, we investigated its role in luminal cell lines. lnc-uc.147 silencing decreased cell viability in both cell lines (CAMA-1 and BT474). lnc-uc.147 silencing also reduced colony formation, number of cells in G2/M during the cell cycle, and increased apoptosis in CAMA-1. These experiments suggests an oncogenic effect of the lnc-uc.147, mainly in luminal A. Moreover, the silencing of the host gene LRBA did not lead to the same cell phenotyping of the lnc-uc.147 silencing.

Understanding lncRNA functions can be accomplished by the identification of lncRNA-bound proteomes [54]. Thinking about that, we performed a pull-down to define the proteins that could interact with the lnc-uc.147 and gain some insight into which molecular pathway and functions. We identify 19 proteins that are precipitated with the lnc-uc.147. Among these protein, 15 (79%) are present also in nucleus, the same sub-cellular localization that lnc-uc.147. Seventeen (90%) of the genes encoding these co-precipitated proteins demonstrated negative or positive correlation with the lnc-uc.147.

There are lncRNAs that specifically bind with one protein and others that bind with multiple proteins [55]. For example, many proteins interact with HOTAIR lncRNA [56–59]. However, the lncRNA lnc-DC seems to interact only with the STAT 3 protein [60]. The lnc-uc.147 apparently have multiple interactions, either direct or indirectly, with several proteins. Overall lnc-uc.147 interact with proteins related to the cellular process involved in cancer development, such as KRT17 [61–63], NACA [64], CALML5 [65], CEP131 [66,67], POM121 [68], and others (Table 2).

Also, it is already known that the lncRNAs may interact with protein complexes [69]. The complexes are mostly formed by RNA binding proteins (RBP) [54]. The ribonucleoproteins in those complexes may control the availability and function of lncRNAs in various steps from RNA biogenesis to RNA metabolism. The cells take advantage of these interactions in forming subcellular structures and regulating the activity of various proteins with functions unrelated to RNA processing. Other interest of these cell is to help the formation of subcellular structures and membrane organelles [70].

Through the STRING website, we could see that proteins binding to lnc-uc.147 form two networks: one between RHOA and CIT, and another among six proteins (WDR18, TEX10, PES1, NOP53, DKC1, and ZC3HAV1). We hypothesize that the lnc-uc.147 could be a scaffold for these two complexes.

The interaction between RHOA and CIT was the only interaction already described in the literature. CIT regulates cytokinesis at a step after RHOA in the contractile process. CIT maintains RHOA localization at the cleavage site, which is necessary for proper RhoA activity and contractile ring dynamics at the end of cytokinesis [71,72]. The lnc-uc.147 could be acting as a guide for these two proteins during cell division.

Another network is formed by the interaction of WDR18, TEX10, PES1, NOP53, DKC1, and ZC3HAV1 proteins and the lnc-uc.147. These proteins participate of RNA processing and regulation [73–79]. Disruptions in RNA regulation have consequences for the cell as it is seen in developmental defects and diseases including cancers in humans where the underlying cause involve RBPs or their target sites within RNAs [80–86].

lncRNAs may act as multiple function in human cells [87], and as lnc-uc.147 has been described for the first time at the present study, the exact mechanisms of action must be deeply investigated in future studies. But, trying to hypothesize other possible mechanisms for this lnc-uc.147, we also analysed the potential cis-acting regulation.

lnc-uc.147 expression was found correlated with the neighbour genes, including *MAB21L2*, *TACR3*, *CXXC4*, *CDKL2* and *G3BP2* with a positive correlation, while *HNRPD*, *HNRPD* and *DCLK2* demonstrated a negative correlation. A positive

correlation was also found between lnc-uc.147 and the two downstream UCRs uc.148 and uc.149.

The downstream gene *MAB21L2* is conserved in most species and has a role in eye development [88]. In cancer, this gene was downregulated in APC-mutants adenomas [89]. *TACR3* expression in the nervous system is essential for the proper function of the human reproductive axis and is highly expressed in the female genital tract [90,91]. The TAC receptor 3 is strongly regulated by oestrogens, and its activation leads to nuclear translocation affecting chromatin structure and gene expression [90]. Alterations in *TACR3* expression are associated with vasomotor symptoms of menopause and leiomyomas [90,91]. *CXXC4* is another gene related to oestrogen receptor, and lower expression of *CXXC4* is associate with tamoxifen resistance [92]. In addition, *CXXC4* knockdown accelerates cell proliferation in vitro and in vivo and renders breast cancer cells insensitive to tamoxifen, whereas *CXXC4* overexpression inhibits cancer cell growth and increases tamoxifen sensitivity of resistant cells [92]. The correlation of lnc-uc.147 with *TACR3* and *CXXC4* could be related to the oestrogen receptor. One hypothesis is for the lnc-uc.147 and *CXXC4* is the lnc-uc.147 bind to the *CXXC4* and inhibit its expression, which increases the tumour aggressiveness. The *CDKL2* is a well-described epidermal growth factor signalling, which is more expressed in mesenchymal breast cancer cells [93]. In addition, higher methylation of *CDKL2* is associate with HER2+ tumours and poor response to neoadjuvant chemotherapy [94,95]. Increased expression of *CDKL2* and its correlation with lnc-uc.147 is not clear at the moment. The *G3BP2* gene is well associated with stress granule, but it also associate with cancer initiation, EMT, tamoxifen resistance, and target therapy in breast cancer [96–100]. The correlation between *GEBP* and lnc-uc.147 could also be related to the oestrogen receptor due to *G3BP* role in tamoxifen resistance. The *HNRPD* and *HNRPD* code for RNA binding proteins [101]. The *HNRPD*, also known as *AUF-1*, is responsible for the stability of the cyclin-dependent kinase inhibitor p16 (*INK4a*) and p21 (*WAF1*). Both p16 and p21 are involved in various anti-cancer processes, including regulating the critical G1 to S phase transition of the cell cycle, senescence and apoptosis [102–104]. The negative correlation of *HNRPD* with lnc-uc.147 seems to be correlated with a more aggressive phenotype of breast cancer tumours. For last, we have a negative correlation of *DCLK2* with lnc-uc.147. The *DCLK2* is a well-described gene in neuronal activity, and in only one recent study, its higher expression was associated with reduced patient survival in chronic lymphocytic leukaemia [105–107]. So, it is not clear the correlation expression of this gene and the lnc-uc.147.

lnc-uc.147 expression was also negatively correlated with mir-18a and a positive correlation with mir-190. In addition, mir-190 bind to the lnc-uc.147 according to a prediction analysis on miRDB website (<http://mirdb.org/>) [53]. Mir-18 was already demonstrated in EMT in BC and is associated with poor survival in BC [108]. The overexpression of mir-190 suppresses metastasis, inhibits migration and invasion, and enhances endocrine therapy sensitivity in BC [109–111]. The lnc-uc.147 could be acting as a decoy sequestering this miRNA and increasing the BC aggressiveness. This analysis indicates another possible regulatory mechanism of lnc-uc.147 in BC.

Despite all informations, the pull-down results and correlation analysis gave us some clues for lnc-uc.147 mechanisms

and reinforce the relevant lnc-uc.147 role in BC cells. However, more studies of how this lncRNA influence tumorigenesis process is necessary.

We show here in several evidence that neoplastic BC cells exhibit a unique expression profile of T-UCRs, specific uc.147, and uc.193, suggesting a significant role of T-UCRs in the malignant process. In this study, we provide more evidence for the role of T-UCRs in BC. We also characterized the lnc-uc.147, T-UCR that have never been described before, which elucidates one more fraction about these complex regions. Indeed, lnc-uc.147 has an oncogenic effect in the luminal BC cell line and can interact with proteins. Furthermore, uc.147 and uc.193 expression have a potential as a BC prognosis marker in luminal patients.

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Disclosure statement

Dr. Calin is the scientific founder of Ithax Pharmaceuticals.

Author contributions

J.C.O, G.A.C., and E.P.Z. designed this study. E.P.Z, J.C.O, and G.A.C wrote the manuscript. E.P.Z, R.B, T.S.J, A.C.R performed most of the wet-lab experiments. C.I., E.K., and C.M. reviewed and performed most bioinformatical analysis. D.F.G and E.M.S.R.F assisted with interpretation. E.P.Z., J.C.O. and G.A.C analyzed the data. All authors read and approved the manuscript's content.

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