A Novel Long Non-Coding RNA Regulates The Integrin, ITGA2 in Breast Cancer

Tristan J Verhoeff
University of Tasmania Menzies Institute for Medical Research
Adele F Holloway
University of Tasmania School of Medicine
Joanne L Dickinson (jo.dickinson@utas.edu.au)
University of Tasmania Menzies Institute for Medical Research https://orcid.org/0000-0003-4621-1703

Research Article

Keywords: lncRNA, integrins, ITGA2, epigenetic, gene regulation

Posted Date: November 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1026681/v1

Version of Record: A version of this preprint was published at Breast Cancer Research and Treatment on January 31st, 2022. See the published version at https://doi.org/10.1007/s10549-021-06496-x.

Abstract

Purpose

ITGA2 encodes the integrin, a2 which mediates metastatic progression, and is a predictor of poor prognosis and chemoresistance in breast cancer. Decreased ITGA2 promoter methylation is implicated as a driver of increased gene expression in aggressive prostate and pancreatic tumours, however the contribution of altered methylation to ITGA2 expression changes in breast tumours has not been examined.

Methods

ITGA2 gene methylation and gene expression was examined in publicly available breast cancer datasets, and ITGA2 promoter methylation was mapped by targeted bisulphite sequencing analysis in breast tumour cell lines. The expression of a putative regulatory long noncoding RNA (lncRNA) was examined by qPCR and its' functionality was investigated using gene knockdown (antisense oligonucleotides) and over expression analysis in breast cancer cell lines.

Results

In breast tumours and breast cancer cell lines the ITGA2 promoter is largely unmethylated, with gene expression variable in tumour subtypes, irrespective of promoter methylation. A novel lncRNA (AC025180.1;ENSG00000249899), named herein I2ALR, was identified at the ITGA2 gene locus, and was variably expressed in breast tumours and breast cancer cell subtypes. I2LAR knockdown resulted in upregulation of ITGA2 gene expression, whilst over-expression of I2ALR resulted in downregulation of ITGA2 mRNA. Further, examination of two downstream targets of ITGA2 associated with breast tumor stemness and metastasis (*CCND1* and *ACLY*), revealed concomitant gene expression changes in response to *I2ALR* modulation.

Conclusion

I2ALR represents a novel regulatory molecule targeting ITGA2 expression in breast tumours; a finding of significant and topical interest to the development of therapeutics targeting this integrin.

Introduction

Cell surface molecules including integrins are critical for metastasis and invasion. Integrins are adhesion receptors with extracellular and cytoplasmic signalling domains. Integrin ITGA2, or a2 (CD49b), forms the a2β1 collagen type I (col-I) and laminin receptor [1, 2], a key mediator of metastasis and marker of poor prognosis in solid tumors including breast, ovarian and pancreatic cancers [3–6]. In normal breast tissue

a2β1 is expressed in the mammary gland at cell-cell/basement membrane interface [7], and is regulated by oestrogen [8]. Primary breast cancer (BrCa) tumors retain the elevated a2β1 expression and estrogen receptor positive (ER+) status of normal breast tissue, with expression of a2β1 and ER often lost in soft tissue metastases [4]. Luminal/ER+ BrCa cell lines (MCF-7, T-47D or MCF-10A) have been reported to express elevated *ITGA2*, relative to basal-metastatic/ER− lines (MDA-MB-231 and MDA-MB-436) [9–11]. However, MDA-MB-231 cells cultured on bone matrix highly express a2 relative to MCF-7 and T-47D cells [12], and preferentially metastasize to bone, where they express elevated a2 [13]. In addition, a2β1 expression promotes stem-cell like behaviour in triple negative BrCa [14].

Epigenetic modifications facilitate the nuanced control of gene expression and play an important role in the regulation of integrins. DNA-methylation, histone modifications, micro RNAs and long non-coding RNAs (LncRNAs) co-ordinate to deliver precise expression of genes, and present exciting chemotherapeutic options. MiRNAs including miR-373-3p and miR-206 are reported to post-transcriptionally regulate ITGA2 protein levels in BrCa [11, 14]. The ITGA2 promoter includes a CpG island (CpGI) which in normal prostate tissues and pancreatic cancer is hypomethylated and associated with upregulated *ITGA2* expression. Methylation at the promoter is increased in the locally invasive LNCaP PrCa cell line which express low levels of *ITGA2*. Whilst in bone metastatic PC3 cells, decreased promoter methylation is associated with high α2β1 expression [3, 15], reflected in PrCa bone metastases [16]. Histone modifications also regulate *ITGA2* [17], and it is hypothesised that a combination of these epigenetic mechanisms contribute to the expression of this gene in different tumor tissue contexts.

In the present study, we demonstrate that methylation at the ITGA2 promoter CpGI in BrCa cells was not associated with changes in *ITGA2* expression, in contrast to previous observations in PrCa, suggesting alternative regulatory mechanisms are at play. Examination of the ITGA2 promoter flanking regions revealed a previously uncharacterized lncRNA, herein named *I2ALR* (AC025180.1, ENSG00000249899, also referred to as lnc-MOCS2-1). This study provides insight into the tissue-specific differential epigenetic mechanisms regulating *ITGA2* and provides important context to current efforts to develop ITGA2-targeted therapeutics.

Materials And Methods

Cell Culture

Cell lines employed for this study included the BrCa cell lines MDA-MB-231, MDA-MB-453, MCF-7 and T-47D and obtained from the American Tissue Cell Collection (ATCC, USA) and the PrCa cell lines, PC3 and LNCap obtained from Cell Bank Australia (CMRI, Sydney). Cell passaging conditions are described in the Supplementary Methods including a description of cell line characteristics (Supplementary Methods Table 1). Cell lines were verified by DNA fingerprinting performed by the Australian Genome Research Facility (May 2021).

Quantitation of Gene Expression

Cell pellets were obtained and RNA was extracted using the RNeasy® Mini Kit (QIAGEN) and converted to cDNA using the iScript™ Reverse Transcription Supermix kit (Bio-Rad Laboratories Inc.). Alternatively, SuperScript® III First-Strand Synthesis Kit (Invitrogen, Thermo Fisher Scientific) was used to specifically detect poly-A transcripts, using an oligo$(dT)_{20}$ primer, and RNase H. Expression levels of genes of interest were quantitated using quantitative PCR and levels are expressed relative to the housekeeping gene *GAPDH*. Primer sequences are presented in Supplementary Methods Table 2. Analyses were performed in triplicate using SensiFAST™ SYBR® No-Rox (Bioline). Thermal cycling conditions are presented in Supplementary Methods Table 2. Standard curves were generated to determine absolute gene expression. Gel electrophoresed PCR products were extracted with QIAquick® Gel Extraction Kit (QIAGEN®).

Methylation analysis of ITGA2 CpGI

To map CpGI methylation at the ITGA2 promoter, gDNA was extracted from cell pellets using DNeasy Blood & Tissue kit (QIAGEN), and bisulphite converted with the EZ DNA Methylation-Gold™ Kit (ZYMO Research). Amplified regions of the bisulphite converted promoter (see Supplementary Methods Table 3 for primer sequences) were generated using MyTaq™ HS Mix (Bioline). Fragments were ligated with pGEM®-T Easy Vector System I (Promega) and transformed used SoloPack® Gold Competent Cells (Agilent Technologies). Ten bacterial colonies were selected per amplified region, cultured and DNA extracted with the QIAprep® Spin Miniprep Kit (QIAGEN).

DNA sequence was obtained using an ABI 3500 Genetic Analyzer (Applied Biosystems), and analysed using Sequencher® software, v4.10.1 (Gene Codes Corporation), and converted into bubble maps of the CpG sites using BiQ Analyzer software (V2.0, Max Planck Insitut fuer Informatik) and Microsoft EXCEL 'CpG Bubble Chart Generator' (V.20061209 alpha, Mark A. Miranda).

Knockdown of lncRNA I2ALR

Knockdown of lncRNA *I2ALR* in MDA-MB-453 cells was achieved using antisense oligonucleotide (ASO) gapmers. Two targeting ASOs and a scrambled control (ASO-Scr) were designed and synthesised by Integrated DNA Technologies (Supplementary Methods Table 4). ASOs were transfected into cell lines using the INTERFERin® *in vitro* siRNA/miRNA transfection reagent protocol (Polyplus-Transfection), at final optimized ASO concentration of 10 nM (in 10.5 mL of media), cells at ~50% confluency and cultured for 24 and 48 hrs before harvesting.

Overexpression of lncRNA I2ALR

To over-express *I2ALR*, a construct, named $I2ALR^{construct}$, was synthesised and cloned into pUC57-Kan by GENEWIZ Inc. (USA). The $I2ALR^{construct}$ contained the combined exons for *ENST00000505701.5* and *ENST00000503559.1*, including an uncharacterized 3' region (U3R) plus the poly-A signal, and 100 bps of polyadenylation following the poly-A signal ($I2ALR^{construct}$; Supplementary Methods Table 5). Using NheI-HF and XhoI restriction enzyme sites incorporated at 5' and 3' flanks respectively, the $I2ALR^{construct}$ fragment was ligated into pcDNA™3.1$^{(+)}$ mammalian expression vector (Invitrogen) and transformed

into SoloPack Gold Competent *E. coli* cells. PCR screening using 'I2ALR set 5' primers (Supplementary Methods Table 2) identified positive colonies which were cultured, and plasmid isolated for subsequent transfection. MCF-7 cells were selected for over-expression experiments as they had moderate *ITGA2* and *I2ALR* expression. Cell lines were transfected by electroporation (5.0×10^6 cells per cuvette + 10 µg of plasmid, voltage 230V, 950 µF, ∞ Ω, distance 4.0 mm). Cells were harvested after 48 hrs.

Publicly Available Datasets

Data for ITGA2 mRNA expression and *ITGA2* methylation were obtained from cBioPortal for Cancer Genomics (https://www.cbioportal.org/) [18]. Datasets: "Breast Invasive Carcinoma (TCGA, Cell 2015)" [19], "Breast invasive carcinoma (TCGA, Firehose Legacy)" Broad Institute [20], "Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS 2019)" [21], "Prostate Adenocarcinoma (*TCGA, Cell 2015*)" [22].

Bioinformatic analysis of the I2ALR lncRNA

Prediction of lncRNA cellular localization used LncLocator (Shanghai Jiao Tong University, http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/) [23]. Interactions between lncRNA and mRNA were predicted with IntaRNA 2.0 bioinformatics tool (V. 4.5.10, Freiburg RNA Tools, http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp) [24]. The secondary structure of lncRNA was predicted with RNAfold (v. 2.4.13, University of Vienna, http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) [25]. Analysis of overall and disease free survival used GEPIA (Zhang Lab, Peking University, http://gepia.cancer-pku.cn/), with comparison of high and low expression groups using Log-rank test (Mantel-Cox test) and high and low cohort group cut-off thresholds being quartile (Cut-off High 75%, Cut-off Low 25%) [26].

Statistical Analysis

Comparisons of the differences was conducted with Student's *t*-tests or one-way ANOVA. Student's *t*-tests were conducted for comparison of two groups or for when equal variance (homoscedasticity) was appropriate. One-way ANOVA analysis was conducted in Rstudio, with Tukey's post-hoc testing on the ANOVA models. All reported p-values are two tailed.

Results

ITGA2 expression is variable in BrCa and PrCa cell lines

Expression of ITGA2 mRNA was examined in cell lines representing different subtypes of BrCa. Expression in MDA-MB-231 cells was 16-fold higher compared with the lowest expressing MDA-MB-453 cells. T-47D and MCF-7 cells displayed intermediate expression (Figure 1A). Differential expression is also observed between PrCa cell lines with bone metastasis derived PC3 cells showing elevated *ITGA2* expression relative to lymphnode metastasis derived LNCaP cells (Figure 1A, with significant expression differences shown) (one-way ANOVA, Tukey's post-hoc tests, $P < 0.05$).

Analyses of publicly available datasets revealed that reduced *ITGA2* expression was a significant predictor of distant metastases (Student's *t*-test, $P < 0.05$, Figure 1B) in BrCa. Expression of *ITGA2* in BrCa and PrCa was elevated in primary tumors and bone metastases and reduced in soft tissue metastases. Statistical significance was evident between the primary tumors and soft tissue metastases in BrCa datasets (Student's *t*-test: $P < 0.001$, Figure 1C; $P < 0.01$, Figure 1D), although statistical comparisons were impacted for the BrCa bone metastasis by the small sample size available. In the PrCa dataset, *ITGA2* expression differences between bone metastases compared to soft tissue metastases were significant (Student's *t*-test, $P < 0.01$, Figure 1E).

DNA methylation of the ITGA2 promoter CpGI

Methylation mapping of the ITGA2 promoter in BrCa cell lines by bisulphite sequencing revealed hypomethylation irrespective of the level of *ITGA2* expression (Figure 2A). In the BrCa dataset (TCGA Cell 2015), ITGA2 promoter methylation was elevated in BrCa soft tissue metastases and lower in both bone metastases and primary tumors (Figure 2B), although not statistically significant. ITGA2 promoter methylation was only weakly inversely associated with expression in the BrCa samples (TCGA Cell 2015) (Figure 2C). However, a stronger correlation was observed in PrCa samples (TCGA Cell 2015) (Figure 2D). Interestingly, the majority of PrCa samples showed high overall ITGA2 promoter methylation (methylation β-scores > 0.6, and none with scores < 0.4). By comparison, promoter methylation varied across the full spectrum at the ITGA2 promoter in BrCa samples. Statistical analysis revealed these differences between BrCa and PrCa to be significant (Student's *t*-test, $P < 0.001$). These data suggest that alternative regulatory mechanisms may be at play.

Expression of lncRNA I2ALR in BrCa and PrCa

In seeking to understand alternative mechanisms employed by BrCa and PrCa cells for regulation of the ITGA2 gene, the *ITGA2* locus was examined, revealing an uncharacterised lncRNA transcribed in reverse, downstream of the ITGA2 transcription start site. Given that lncRNAs are known to regulate genes in *cis*, we sought to examine whether this novel lncRNA regulated *ITGA2*. The uncharacterised lncRNA gene AC025180.1 (ENSG00000249899), was designated ITGA2 Antisense LncRNA (*I2ALR*). *I2ALR* expression assessed by qPCR was relatively low compared to *ITGA2* expression, and of the examined BrCa cell lines, was predominantly expressed by MDA-MB-453 cells, followed by T-47D and MCF-7, and then MDA-MB-231 with the lowest expression. Expression in the PrCa cell lines PC3 and LNCaP is presented for comparison (Figure 3A). In BrCa cell lines *I2ALR* expression levels were weakly inversely correlated with levels of ITGA2 mRNA (Figure 3B). In contrast, and somewhat surprisingly, in PrCa cell lines *I2ALR* expression was highest in PC3 cells (Figure 3A) in which *ITGA2* is also highly expressed (Figure 1A).

Knockdown of I2ALR increases ITGA2 expression

To determine whether modulation of *I2ALR* influenced *ITGA2* expression, antisense oligonucleotides (ASOs) were designed to target the lncRNA (Supplementary Results Figure 1A). Transient transfection of ASO-160 and ASO-991, targeting regions of *I2ALR*, into MDA-MB-453 cells at increasing concentrations (10 nM, 25 nM and 50 nM) revealed both ASOs induced *I2ALR* knockdown relative to the ASO scrambled

control (ASO-Scr) after 24-hours. *I2ALR* knockdown was clearly evident at the lowest concentration (10 nM) and this concentration was therefore selected for subsequent experiments (minimising possible off target effects at higher concentrations; data shown Supplementary Results Figure 1B,C).

Transfection of MDA-MB453 cells with ASO-160 and ASO-991 at 10nM revealed effective knockdown of *I2ALR* (Figure 4A; Student's *t*-tests, P<0.05; one-way ANOVA, P = 0.055) and an observed increase in *ITGA2* mRNA levels (Figure 4B; one-way ANOVA, Tukey's post hoc tests, P<0.05). *I2ALR* knockdown was also observed at 48-hours (Supplementary Figure 1D) with concomitant significant upregulation of *ITGA2* expression (Supplementary Figure 1E).

Overexpression of the putative I2ALR transcript reduces ITGA2 expression

To further confirm an *I2ALR* regulatory effect on *ITGA2*, an I2ALR over-expression construct, $I2ALR^{construct}$ was transfected into MCF-7 cells. High expression of $I2ALR^{construct}$ was confirmed in transfected cells compared with the vector control and untreated cells (Figure 4C; one-way ANOVA; Tukey's post-hoc tests, P<0.001). Expression of *ITGA2* was reduced by over-expression of *I2ALR* relative to vector and un-transfected controls (Figure 4D; one-way ANOVA, Tukey's post-hoc tests, P<0.001).

Effect of I2ALR on downstream ITGA2 targets

In order to determine whether I2ALR-mediated knockdown of ITGA2 impacted downstream genes, the expression of two known ITGA2-responsive genes, CCND1 and ACLY was examined. Increased ITGA2 expression upregulates these genes promoting stemness and metastatic progression in BrCa [14]. Both *ACLY* and *CCND1* were downregulated by over-expression of *I2ALR* in MCF-7 cells. A statistically significant decrease in *ACLY* expression was observed when compared with controls (P < 0.05, one-way ANOVA Tukey's post-hoc test). A smaller decrease in *CCND1* expression also observed (P < 0.05, Student's *t*-test, although non-significant using one-way ANOVA) (Figure 4E–F).

In a second breast cancer cell line, targeted KD of *I2ALR* by ASO-160 in MDA-MB-453 cells (associated with increased *ITGA2* expression) was also observed to result in a concomitant increase in expression of *ACLY* and *CCND1* (P<0.05; one-way ANOVA, Tukey's post-hoc test) (Figure 4G–H) at 48 hours. These results are consistent with ITGA2-dependent regulation of *ACLY* and *CCND1* reported by Adorno-Cruz *et al.* (2021)[14].

Characterisation of I2ALR

A diagrammatic representation of the I2ALR in relation to the ITGA2 promoter is shown in Figure 5A showing two putative TSSs. The DNA sequence is shown in Figure 5B, with additional analysis using alternative primer pairs revealing that the classical poly-A signal is the likely preferred motif to the alternative poly-A signal (see Supplementary Results Figure 2 for additional analsyses). Analysis of lncRNA localization using LncLocator predicts subcellular localizations based on lncRNA sequence. Analysis of the combined exons of *ENST00000505701.5* and *ENST00000503559.1* indicated that *I2ALR*

was predicted to be translocated to the cytoplasm (57%), and inclusion of the uncharacterized 3'- UTR did not substantially alter this prediction (55%). The predicted localization of I2LAR was also consistent with experimental cell line data available in the LncExpDB database for this transcript (*HSALNG0041756*) [27].

Given that lncRNAs are known to form lncRNA-[RNA binding protein]-mRNA complexes, putative interactions were investigated *in silico* between the 1112 bp I2ALR^{construct} sequence and *ITGA2* mRNA using the IntaRNA 2.0 bioinformatics tool. Interactions with a seed sequence of > 6 ideal base-pairings were considered, yielding five energetically favourable pairings (Supplementary Results Figure 3A). The lncRNA secondary structure was also predicted with RNAfold (Supplementary Results Figure 3B), with two hairpin loop domains apparent and a minimum free energy of −319 kcal/mol.

I2ALR expression and cancer survival

To examine whether *I2ALR* is associated with clinical outcome, expression of *I2ALR* was examined in publicly available cancer datasets. Analysis of GEPIA data revealed elevated *I2ALR* expression (normalized to *GAPDH*) in BrCa (breast invasive carcinoma) was associated with improved overall survival post diagnosis. However, in later stages of disease this effect was no longer evident and was not statistically significant (Figure 5C). In keeping with previous findings there was little correlation with overall survival in the PRAD (prostate adenocarcinoma) dataset (Figure 5E). Examination of other tumor types revealed higher *I2ALR* expression correlated with improved overall survival (Figure 5D and F).

Discussion

Several studies have now shown that multiple epigenetic mechanisms regulate *ITGA2* expression in solid tumors including altered promoter methylation and post transcriptional regulation by miRNAs [11, 28]. In prostate tumors *ITGA2* expression is highly correlated with promoter hypermethylation [15], however here it was shown that in breast tumors the promoter is largely hypomethylated, yet *ITGA2* expression remains highly variable. Similarly, BrCa cell lines exhibit hypomethylation at the ITGA2 promoter irrespective of expression, consistent with a recent report [29]. Here we describe a novel lncRNA (*I2ALR*) which is variable expressed in BrCa cell lines and functions in BrCa cells to regulate ITGA2 gene expression. Further we provide evidence that *I2ALR* activity may be cell-type specific, as its expression did not correlate with *ITGA2* expression in prostate tumor cells.

Adorno-Cruz *et al.* (2021)[14] have recently reported *ITGA2* expression in different breast tumours with high expression associated with reduced survival, and the significance and magnitude of this effect varies between BrCa sub-types. Here we accessed GEPIA data which showed that higher *I2ALR* expression was associated with improved survival, up to approximately 120 months, over a similar time frame as that reported by Adorno-Cruz *et al.* (2021)[14]. We were not able to examine *I2LAR* expression in breast tumor sub-types using the GEPIA data, as it did not permit subclassification based on breast tumor sub-type or stage. This may have impacted the ability detect a significant association with survival. More in depth examination of a role for this lncRNA in breast cancer sub-types is warranted. For other cancers,

GEPIA data shows that *I2ALR* expression was significantly associated with improved survival in several other solid tumor types.

High levels of *ITGA2* expression has been previously associated with stemness and the initiation of metastases in both prostate and breast tumor cells [30]. Recent studies have shown that ITGA2 induced proliferative and metastatic effects are mediated by the metabolic gene ACLY [14]. ACLY is an enzyme involved in fatty acid synthesis, and has an established role in modulation of proliferation, migration and apoptosis in many tumor types including breast cancer [31]. Similarly, overexpression of the CCND1 gene, a key regulator of cell cycle, is frequently observed in breast tumors. *I2ALR* overexpression and knockdown experiments resulted in reduced and increased *ITGA2* expression respectively with concomitant expected changes in ACLY and CCND1 gene expression observed in both MCF7 and MDA MB453 cells. These changes were consistent with decreased expression of *ACLY* and *CCND1* in MDA MB231 cells following siRNA knockdown of ITGA2 and provide strong supportive evidence for a role for this lncRNA in *ITGA2* regulation in breast cancer cells.

Although lncRNAs are known to guide epigenetic modifications [32, 33], our data indicates a direct interaction with ITGA2 mRNA may be occurring given a suppressive effect of *I2ALR* was observed in 24 hours. The *I2ALR* TSS is downstream of ITGA2's promoter CpG island, possibly allowing differential regulation of the lncRNA in the presence of the methylated ITGA2 promoter. Deregulation, or sequestering of *I2ALR*, may permit the increase of *ITGA2* expression required for bone metastasis. Like *I2ALR*, other antisense lncRNAs also regulate expression of their adjacent gene; e.g. *HOTTIP* repressing *HOXA13* [34], and *BLNCR* regulating *ITGB1* [35]. *I2ALR* was found to comprise 5 exons, with a polyadenylated extended terminal exon (U3R). We hypothesise *I2ALR* forms a lncRNA-[RNA binding protein]-mRNA complex, destabilizing ITGA2 mRNA, in a similar manner to the lncRNAs, *RP11* and *HOXA11-AS*. These lncRNAs bind cytoplasmic hnRNPA2B1 and STAU1 proteins, and mRNAs *FBXO45*/ *SINH1* and *KLF2* respectively. The lncRNA-mRNA complementary interactions guide specificity in the case of *RP11*, to facilitate degradation of their target mRNAs [36, 37]. *In silico* analyses of *I2ALR* support this proposed mechanism; predicted cytoplasmic localization (as well as experimentally validated expression) was consistent with similar lncRNAs (e.g. *LINC01354* [38]) and complementary regions between ITGA2 mRNA and *I2ALR* were predicted. Interestingly one of these predicted complementary interactions was in the ITGA2 mRNA 5'-UTR while the remaining four were all within the 3'-UTR, regions typically engaged by regulatory RNAs such as microRNAs. The lncRNA *RP11* downregulated SIAH1 and FBXO45 mRNAs by complementary interactions within their CDS and 3'UTR regions [36], and *ITGB2-AS1* downregulated ITGB2 mRNA via an interaction in the mRNAs 5'UTR [39].

The α2 integrin has been targeted by small molecules and antibodies as potential therapies for cancer and other diseases, some reaching phase II trials [40, 41]. There is also interest in lncRNAs as potential novel therapeutics, and the FDA has approved ASO drugs targeting mRNA transcripts to degrade them via RNase H, or as inhibitors of translation [42]. Furthermore, lncRNAs are potential biomarkers of disease, for example *prostate cancer antigen 3* in PrCa [43], and *HOTAIR* as a marker of chemotherapy resistance [44].

Our limited understanding of mechanisms controlling the complex temporal and context dependent expression of the ITGA2 gene has may have hampered the ability to test prospective therapeutics in appropriate contexts. Identification of this lncRNA and its potential role in ITGA2 gene regulation is an important step forward as it offers novel insight into the cell type and context dependent mechanisms controlling α2 integrin expression in tumor development. These findings shed light on the role of *ITGA2* and the nuanced understanding needed for the development and testing of therapeutics targeting this integrin.

Declarations

Statements and Declarations: The authors declare they have no conflicts of interest.

Funding: This study was supported by the Andree Greenwood Secondary Breast Cancer Fund; the Hobart Police Charity Trust; and the Royal Hobart Hospital Research Foundation. JLD is supported by a Select Foundation Cancer Research Fellowship, and previously supported by an Australian Research Council Future Fellowship.

Author contributions

JLD and AFH conceived and designed the work, TJV performed laboratory experiments, analysed the data. TJV prepared the original draft of the work. TJV, JLD and AFH interpreted the data, and participated in revisions and editing of the manuscript.

Acknowledgements

The authors would like to convey their sincere thanks to participants of the Tasmanian Familial Prostate Cancer study participants who have generously consented to participate in this study and allow their stored pathology samples to be used for the purposes of this research. The authors would like to acknowledge Dr Kelsie Raspin provided cDNA samples for PrCa cell lines.

Data Availability Statement

All data generated or analysed during this study are included in this published article and its supplementary information files. Publicly available data that support the findings of this study are openly available https://www.cbioportal.org/

References

1. Takada Y, Ye X, Simon S: **The integrins**. *Genome Biology* 2007, **8**(215).
2. Zeltz C, Gullberg D: **The integrin-collagen connection - a glue for tissue repair?** *Journal of Cell Science* 2016, **129**:653-664.

3. Nones K, Waddell N, Song S, Patch A, Miller D, Johns A, W J, Kassahn K, Wood D, Bailey P *et al*: **Genome-wide DNA methylation patterns in pancreatic ductal adenocarcinoma reveal epigenetic deregulation of SLIT-ROBO, ITGA2 and MET signaling**. *International Journal of Cancer* 2014, **135**(5):1110-1118.
4. Ramirez NE, Zhang Z, Madamanchi A, Boyd KL, O'Rear LD, Nashabi A, Li Z, Dupont WD, Zijlstra A, Zutter MM: **The α2β1 integrin is a metastasis suppressor in mouse models and human cancer**. *The Journal of Clinical Investigation* 2011, **121**(1):226-237.
5. Ma L, Sun Y, Li D, Li H, Jin X, Ren D: **Overexpressed ITGA2 contributes to paclitaxel resistance by ovarian cancer cells through the activation of the AKT/FoxO1 pathway**. *Aging* 2020, **12**(6):5336–5351.
6. Ren D, Zhao J, Sun Y, Li D, Meng Z, Wang B, Fan P, Liu Z, Jin X, Wu H: **Overexpressed ITGA2 promotes malignant tumor aggression by up-regulating PD-L1 expression through the activation of the STAT3 signaling pathway**. *Journal of Experimental & Clinical Cancer Research* 2019, **38**(485).
7. Zutter MM, Santoro SA: **Widespread Histologic Distribution of the α1β1 Integrin Cell-surface Collagen Receptor**. *American Journal of Pathology* 1990, **137**(1):113–120.
8. Drabovich AP, Pavlou MP, Schiza C, Diamandis EP: **Dynamics of Protein Expression Reveals Primary Targets and Secondary Messengers of Estrogen Receptor Alpha Signaling in MCF-7 Breast Cancer Cells**. *Molecular & Cellular Proteomics* 2016, **15**(6):2093-2107.
9. Maemura M, Akiyama SK, Woods Jr. VL, Dickson RB: **Expression and ligand binding of α2β1 integrin on breast carcinoma cells**. *Clinical & Experimental Metastasis* 1995, **13**(4):223-235.
10. Lichtner RB, Howlett AR, Lerch M, Xuan J-A, Brink J, Langton-Webster B, Schneider MR: **Negative Cooperativity between α3β1 and α2β1 Integrins in Human Mammary Carcinoma MDA MB 231 Cells**. *Experimental Cell Research* 1998, **240**:368–376.
11. Ding W, Fan X-L, Xu X, Huang J-Z, Xu S-H, Geng Q, Li R, Chen D, Yan G-R: **Epigenetic Silencing of ITGA2 by MiR-373 Promotes Cell Migration in Breast Cancer**. *PLOS One* 2015, **10**(8).
12. van der Pluijm G, Vloedgraven H, Papapoulos S, Löwick C, Grzesik W, Kerr J, Robey PG: **Attachment characteristics and involvement of integrins in adhesion of breast cancer cell lines to extracellular bone matrix components.** *Laboratory investigation; a journal of technical methods & pathology* 1997, **77**(6):665-675.
13. Ibaragi S, Shimo T, Hassan NMM, Isowa S, Kurio N, Mandai H, Kodama S, Sasaki A: **Induction of MMP-13 Expression in Bone-metastasizing Cancer Cells by Type I Collagen through Integrin α1β1 and α2β1-p38 MAPK Signaling**. *Anticancer Research* 2011, **32**(4):1307-1313.
14. Adorno-Cruz V, Hoffmann AD, Liu X, Dashzeveg NK, Taftaf R, Wray B, Keri RA, Liu H: **ITGA2 promotes expression of ACLY and CCND1 in enhancing breast cancer stemness and metastasis**. *Genes & Diseases* 2021, **In Press**.
15. Chin SP, Marthick JR, West AC, Short AK, Chuckowree J, Polanowski AM, Thomson RJ, Holloway AF, Dickinson JL: **Regulation of the *ITGA2* Gene by Epigenetic Mechanisms in Prostate Cancer**. *The Prostate* 2015, **75**:723-734.

16. Sottnik JL, Daignault-Newton S, Zhang X, Morrissey C, Hussain MH, Keller ET, Hall CL: **Integrin alpha2beta1 (α2β1) promotes prostate cancer skeletal metastasis**. *Clinical & Experimental Metastasis* 2013, **30**:569-578.
17. Ferraro A, Boni T, Pintzas A: **EZH2 Regulates Cofilin Activity and Colon Cancer Cell Migration by Targeting ITGA2 Gene**. *PLOS One* 2014, **9**(12).
18. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E *et al*: **The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data**. *Cancer Discovery* 2012, **2**(5):401-404.
19. Ciriello G, Gatza ML, Beck AH, Wilkerson MD, Rhie SK, Pastore A, Zhang H, McLellan M, Yau C, Kandoth C *et al*: **Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer**. *Cell* 2015, **163**(2):506-519.
20. Harvard BIoM: **stddata__2016_01_28/data/BLCA/20160128**. In: *Firehouse Broad GDAC.* Broad Institute of MIT & Harvard; 2016.
21. Abida W, Cyrta J, Heller G, Prandi D, Armenia J, Coleman I, Cieslik M, Benelli M, Robinson D, Van Allen EM *et al*: **Genomic correlates of clinical outcome in advanced prostate cancer**. *Proceedings of the National Academy of Sciences of the United States of America* 2019, **116**(23):11428-11436.
22. Cancer Genome Atlas (TCGA) Research Network: **The molecular taxonomy of primary prostate cancer**. *Cell* 2015, **163**(4):1011-1025.
23. Cao Z, Pan X, Yang Y, Huang Y, Shen H-B: **The lncLocator: a subcellular localization predictor for long non-coding RNAs based on a stacked ensemble classifier**. *Bioinformatics* 2018, **34**(13):2185-2194.
24. Mann M, Wright PR, Backofen R: **IntaRNA 2.0: enhanced and customizable prediction of RNA-RNA interactions**. *Nucleic Acids Research* 2017, **45**(1):435-439.
25. Lorenz R, Bernhart SH, zu Siederdissen CH, Tafer H, Flamm C, Stadler PF, Hofacker IL: **ViennaRNA Package 2.0**. *Algorithms for molecular biology* 2011, **6**(26).
26. Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z: **GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses**. *Nucleic Acids Research* 2017, **45**(1):98-102.
27. Li Z, Liu L, Jiang S, Li Q, Feng C, Du Q, Zou D, Xiao J, Zhang Z, Ma L: **LncExpDB: an expression database of human long non-coding RNAs**. *Nucleic Acids Research* 2021, **49**(D1):D962-D968.
28. Augoff K, Das M, Bialkowska K, McCue B, Plow EF, Sossey-Alaoui K: **miR-31 is a broad regulator of β1-integrin expression and function in cancer cells**. *Molecular Cancer Research* 2011, **9**(11):1500-1508.
29. Strelnikov VV, Kuznetsova EB, Tanas AS, Rudenko VV, Kalinkin AI, Poddubskaya EV, Kekeeva TV, Chesnokova GG, Trotsenko ID, Larin SS *et al*: **Abnormal promoter DNA hypermethylation of the integrin, nidogen, and dystroglycan genes in breast cancer**. *Scientific Reports* 2021, **11**(2264).
30. Eaton CL, Colombel M, van der Pluijm G, Cecchini M, Wetterwald A, Lippitt J, Rehman I, Hamdy F, Thalman G: **Evaluation of the frequency of putative prostate cancer stem cells in primary and metastatic prostate cancer**. *The Prostate* 2010, **70**(8):875-882.

31. Chen Y, Li K, Gong D, Zhang J, Li Q, Zhao G, Lin P: **ACLY: A biomarker of recurrence in breast cancer**. *Pathology, Research and Practice* 2020, **216**(9).
32. Zhao Y, Sun H, Wang H: **Long noncoding RNAs in DNA methylation: new players stepping into the old game**. *Cell & Bioscience* 2016, **6**.
33. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai M-C, Hung T, Argani P, Rinn JL *et al*: **Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis**. *Nature* 2010, **464**(1071-1076).
34. Kim D, Song J, Han J, Kim Y, Chun C-H, Jina E-J: **Two non-coding RNAs, MicroRNA-101 and HOTTIP contribute cartilage integrity by epigenetic and homeotic regulation of integrin-α1**. *Cellular Signalling* 2013, **25**(12):2878-2887.
35. Tanis SEJ, Köksal ES, van Buggenum JAGL, Mulder KW: **BLNCR is a long non-coding RNA adjacent to integrin beta-1 that is rapidly lost during epidermal progenitor cell differentiation**. *Scientific Reports* 2019, **9**.
36. Wu Y, Yang X, Chen Z, Tian L, Jiang G, Chen F, Li J, An P, Lu L, Luo N *et al*: **m^6A-induced lncRNA RP11 triggers the dissemination of colorectal cancer cells via upregulation of Zeb1**. *Molecular Cancer* 2019, **18**(87).
37. Liu Z, Chen Z, Fan R, Jiang B, Chen X, Chen Q, Nie F, Lu K, Sun M: **Over-expressed long noncoding RNA HOXA11-AS promotes cell cycle progression and metastasis in gastric cancer**. *Molecular Cancer* 2017, **16**(82).
38. Li J, He M, Xu W, Huang S: **LINC01354 interacting with hnRNP-D contributes to the proliferation and metastasis in colorectal cancer through activating Wnt/β-catenin signaling pathway**. *Journal of Experimental & Clinical Cancer Research* 2019, **38**(1).
39. Liu M, Gou L, Xia J, Wan Q, Jiang Y, Sun S, Tang M, He T, Zhang Y: **LncRNA ITGB2-AS1 Could Promote the Migration and Invasion of Breast Cancer Cells through Up-Regulating ITGB2**. *International Journal of Molecular Sciences* 2018, **19**(7):1866.
40. Adorno-Cruzab V, Liu H: **Regulation and functions of integrin α2 in cell adhesion and disease**. *Genes & Diseases* 2019, **6**(1):16-24.
41. Raab-Westphal S, Marshall JF, Goodman SL: **Integrins as Therapeutic Targets: Successes and Cancers**. *Cancers* 2017, **9**(9):110.
42. Bennett CF: **Therapeutic Antisense Oligonucleotides are Coming of Age**. *Annual Review of Medicine* 2019, **70**:307-321.
43. Wei JT, Feng Z, Partin AW, Brown E, Thompson I, Sokoll L, Chan DW, Lotan Y, Kibel AS, Busby JE *et al*: **Can urinary PCA3 supplement PSA in the early detection of prostate cancer?** *Journal of Clinical Oncology* 2014, **32**(36):4066–4072.
44. Deng H, Zhang J, Shi JJ, Guo ZD, He CR, Ding L, Tang JH, Hou Y: **Role of long non-coding RNA in tumor drug resistance**. *Tumor Biology* 2016, **37**:11623–11631.

Figures

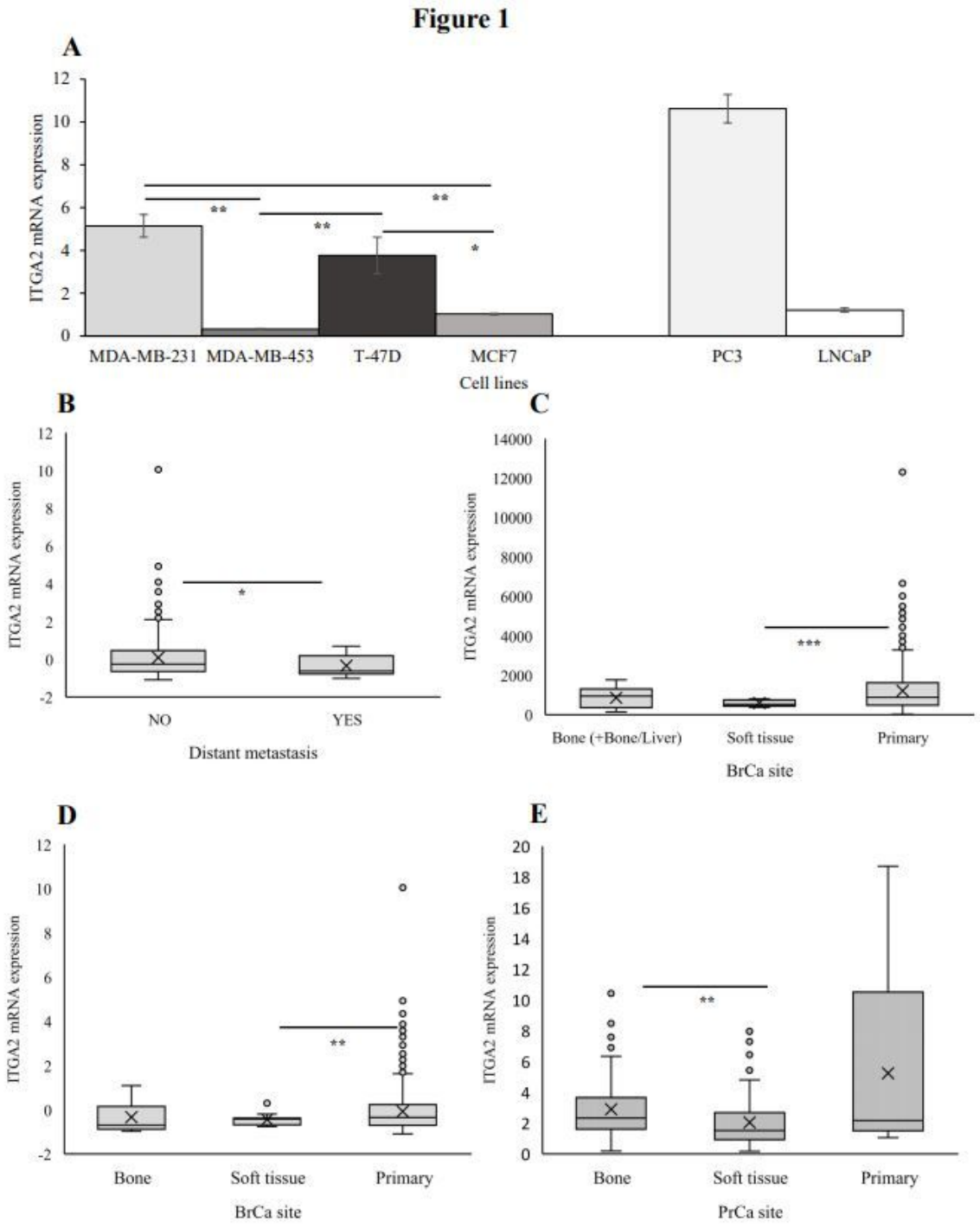

Figure 1

ITGA2 mRNA levels in BrCa, PrCa cell lines and publicly available tumor datasets. (A) Expression of ITGA2 in BrCa and PrCa cell lines shown as absolute copy number normalized to GAPDH, ±SEM (n = 3 biological replicates). (B) ITGA2 expression as predictor of distant BrCa metastasis, TCGA Firehouse Legacy data. (C) ITGA2 mRNA expression in primary tumors relative to bone and soft tissue metastases in BrCa, TCGA Cell 2015 data, (D) Firehouse Legacy data and (E) in PrCa, PNAS 2019 data. (B–E) mRNA

expression = RNA-Seq-V2 RSEM values or FPKM capture values. P-value notation, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

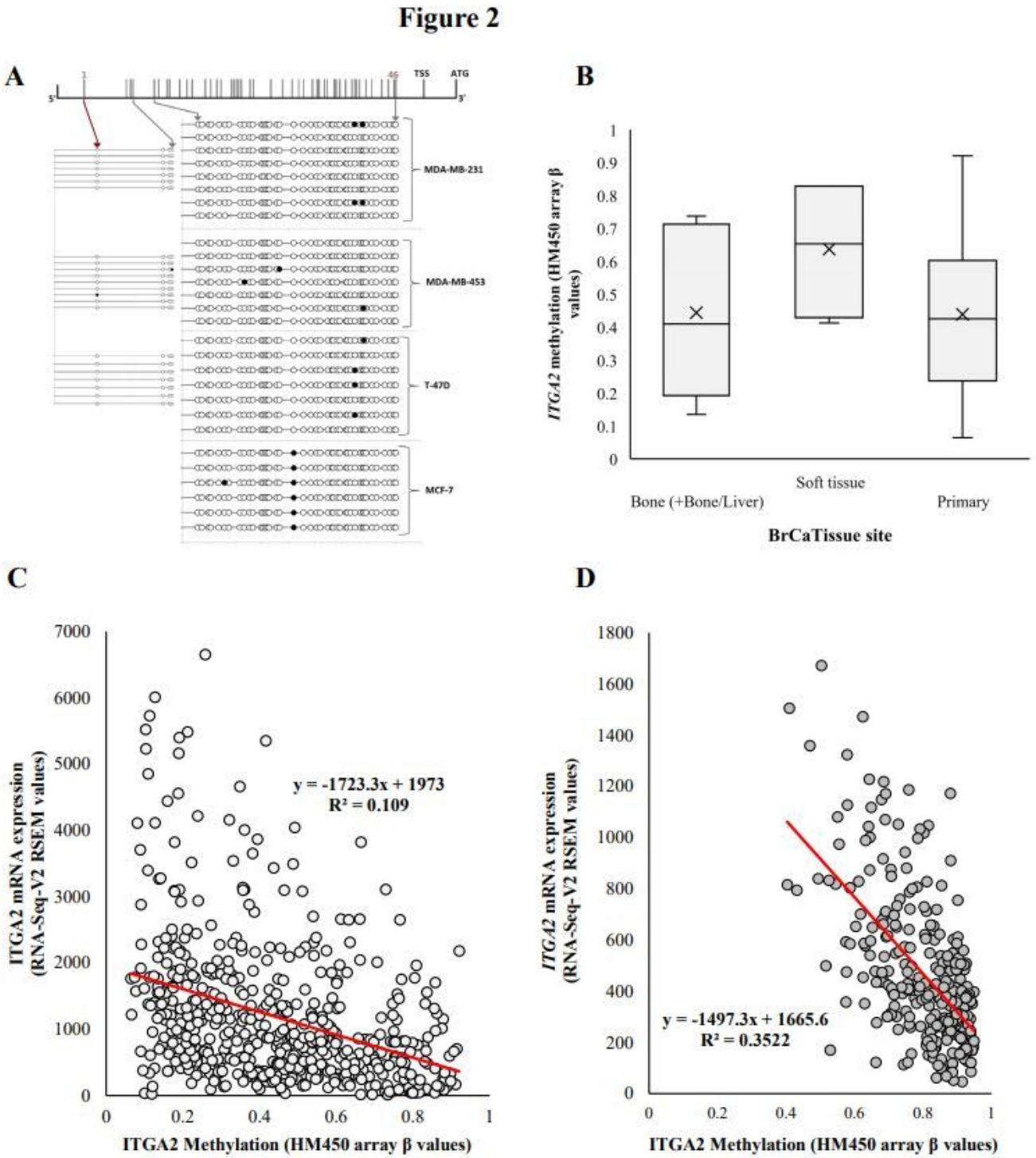

Figure 2

ITGA2 promoter methylation in BrCa and PrCa. (A) From top: diagrammatic representation of the 600 bp ITGA2 promoter comprising a CpGI of 46 CpG sites (vertical grey bars). Diagrammatic representation (Bubble maps) of CpG sites, each line represents a cloned DNA fragment (5'–3'). Open and black circles

represent unmethylated and methylated CpG sites, respectively. CpG sites 1–4 (left panel) and 5–46 (right panel). (B–D) ITGA2 methylation in BrCa and PrCa tumors. (B) Methylation of ITGA2 reported in the public dataset TCGA Cell 2015 for BrCa metastases. (C) ITGA2 mRNA expression versus ITGA2 methylation reported in BrCa dataset TCGA Cell 2015 (n = 551 samples). (D) ITGA2 mRNA expression versus ITGA2 methylation reported in the PrCa dataset TCGA Cell 2015 (n = 289 samples). (C–D) mRNA expression = RSEM values (RNA Seq V2 data). (B–D) methylation levels = β values.

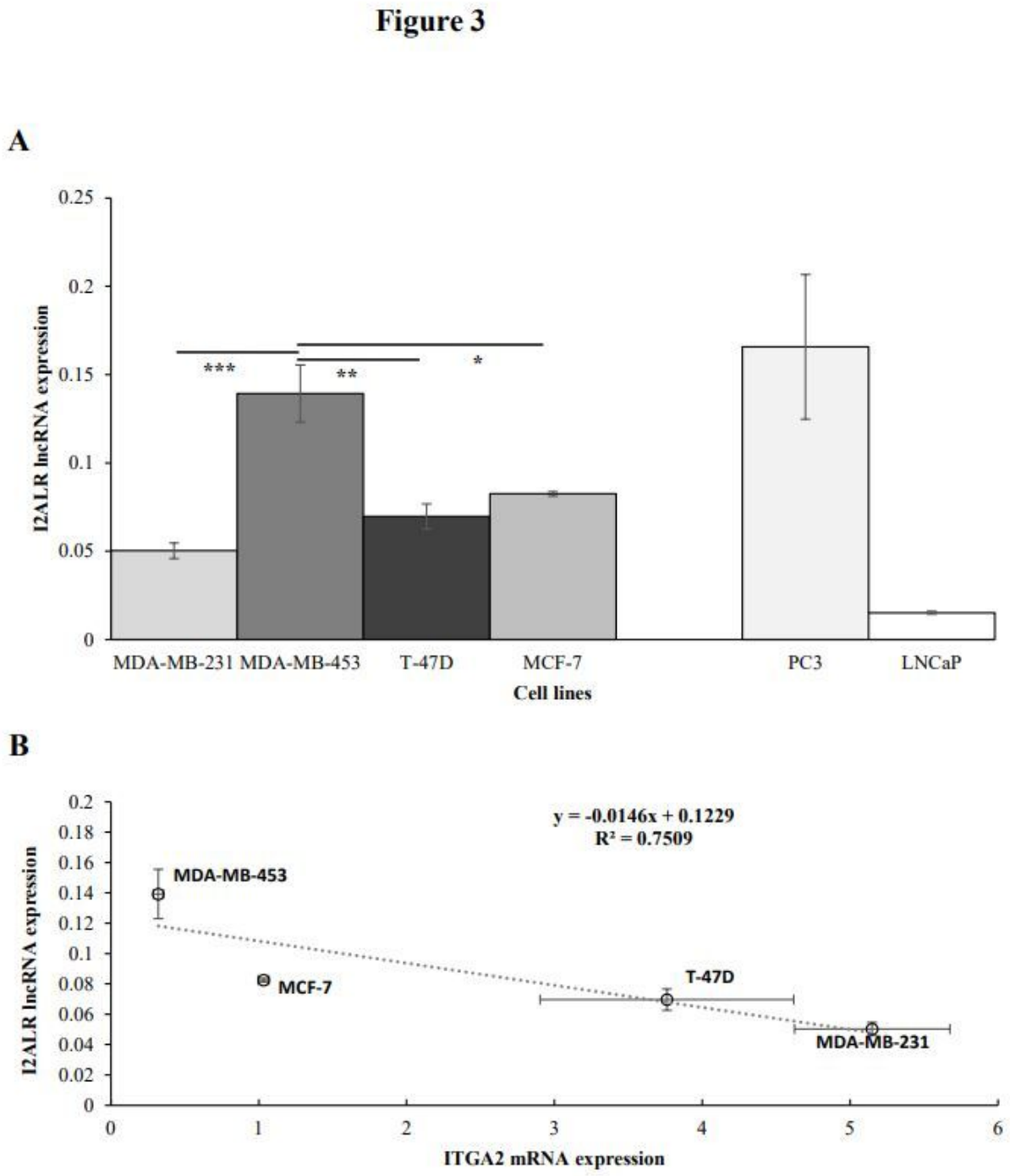

Figure 3

Expression of I2ALR in BrCa and PrCa cell lines in relation to ITGA2 mRNA. (A) I2ALR expression in BrCa and PrCa cell lines. (B) I2ALR expression relative to ITGA2 mRNA expression in the BrCa cell lines (R2 = 0.75). Expression = absolute copy number normalized to GAPDH, ±SEM (n = 3). Statistical significance (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). Statistical tests not performed for PrCa cell line (1 biological replicate); SEM = technical replicates.

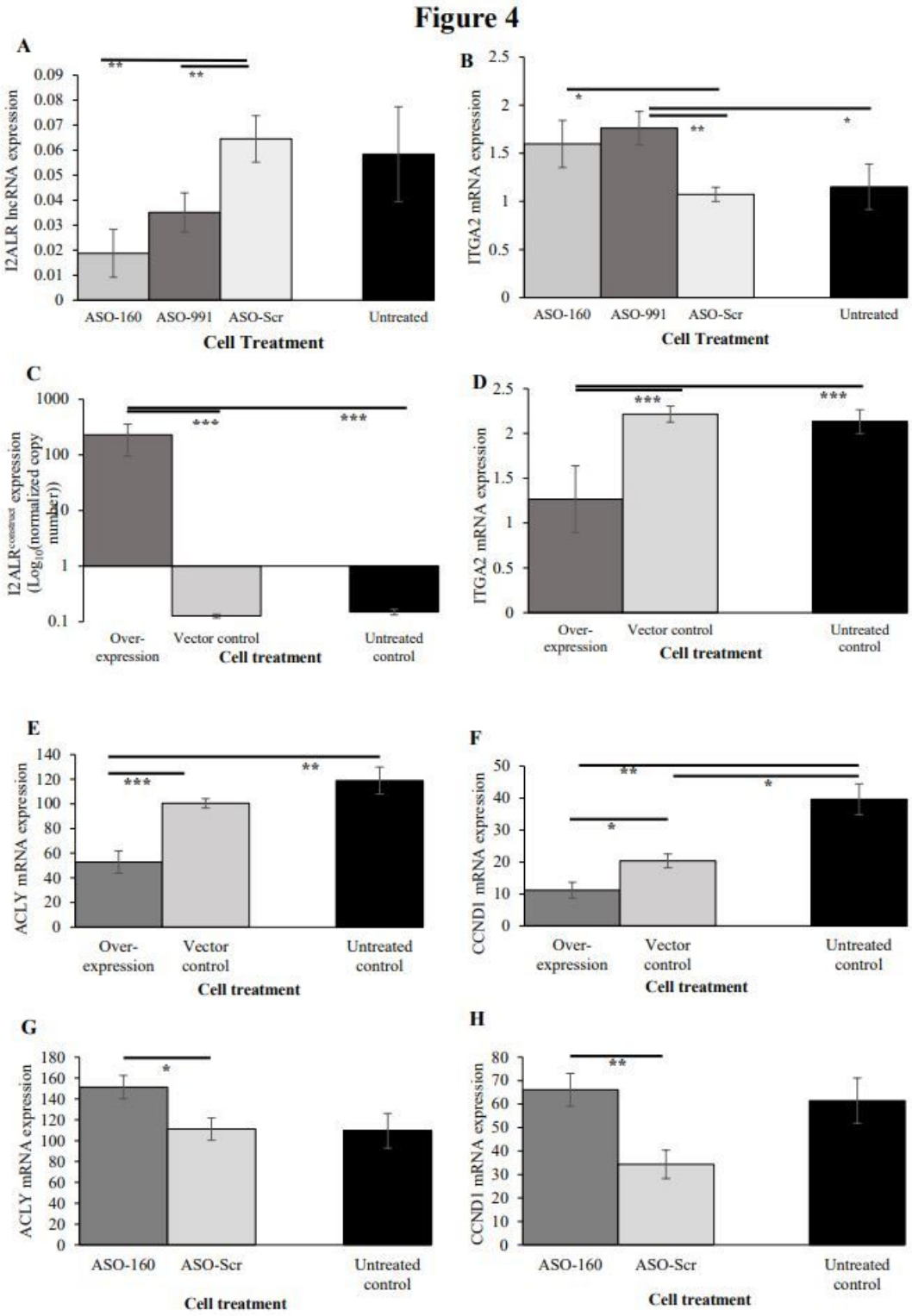

Figure 4

I2ALR lncRNA knockdown (KD) and overexpression effects on ITGA2 and its downstream target genes. (A-B) MDA-MB-453 cells transfected with ASO-160 or ASO-991 targeting I2ALR, or a non-targeting ASO-Scrambled (ASO-Scr) control after 24-hours (final ASO concentrations 10 nM, n = 3 biological replicates, untreated cells included as controls). I2ALR mRNA levels are shown in (A) and ITGA2 mRNA expression in (B). (C–D) MCF-7 cells transfected with the over-expression I2ALRconstruct, a pcDNA3.1+ vector control, or untreated (n = 3 biological replicates). (C) I2ALR overexpression in MCF-7 cells vs. controls, y-axis scale adjusted to log base 10. (D) ITGA2 expression in MCF-7 cells overexpressing I2ALR compared with vector only and untreated controls. (E–F) Expression of ACLY (E), and CCND1 (F) in MCF-7 cells overexpressing I2ALR versus vector only and untreated controls, 48 hours following transfection. (G–H) Expression of ACLY (G) and CCND1 (H) in MDA-MB-453 cells transfected with ASO-160 at 10nM inducing I2ALR knockdown, 48 hours following transfection. RNA levels = absolute copy number normalized to GAPDH, ±SEM (n = 3). Statistical significance *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, one-way ANOVA Tukey's post-hoc test used for all analyses, with the exception of 'F' which was significant for Student's t-test only.

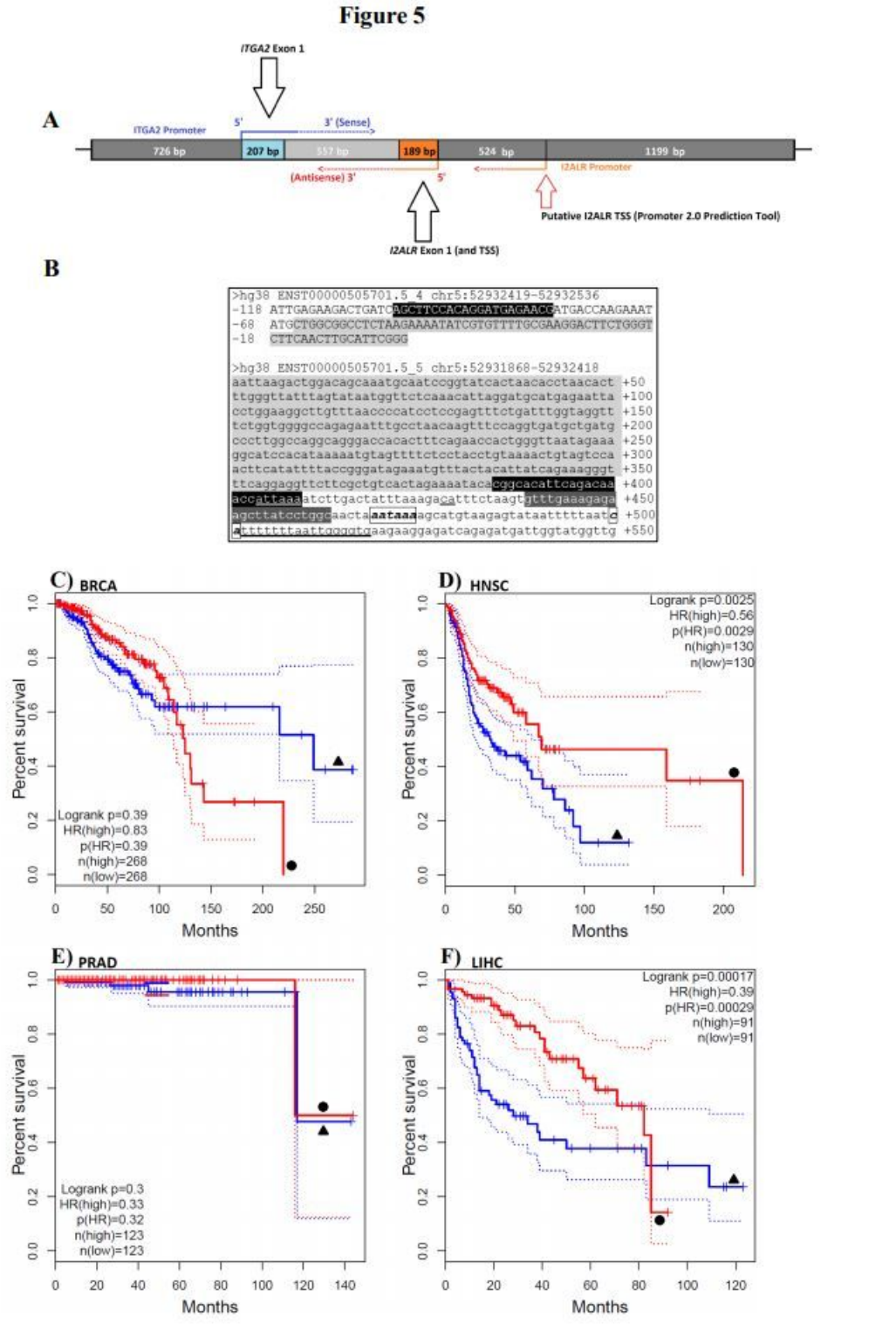

Figure 5

Characterization of the I2ALR and correlation with cancer survival. (A) Diagrammatic representation of the location of I2ALR (5'–3') in relation to the ITGA2 locus displaying two bioinformatically predicted TSS for I2ALR. (B) Amplified I2ALR DNA fragment; confirmed DNA sequence is highlighted in grey with the DNA primers in black. Amplification employing a third primer (highlighted dark grey) suggested that the classical poly-A signal (boxed-bold-italicized text) is preferred to the alternate poly-A signal (underlined

text) data shown in Supplementary Results Figure 2. (C–F) Overall survival using GEPIA data for I2ALR (ENSG00000249899) expression, normalized to GAPDH for (C) breast invasive carcinoma (BRCA), (D) head and neck squamous cell carcinoma (HNSC), (E) prostate adenocarcinoma (PRAD), and (F) liver hepatocellular carcinoma (LIHC). High expression cohort = ‘circle’ and low expression cohort = ‘triangle’, gene expression is expressed as transcripts per million with dotted lines = 95% CI, P-value (Logrank p) and hazard ratio p values (“p(HR)”) displayed, number of samples in each cohort = “n(high)” and “n(low)”.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Verhoeffetal2021SupplementaryMethods271021.pdf
- Verhoeffetal.SuppResults271021.pdf