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Association of invasion-promoting tenascin-C additional domains with breast cancers in young women

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Abstract

Introduction: Tenascin-C (TNC) is a large extracellular matrix glycoprotein that shows prominent stromal expression in many solid tumours. The profile of isoforms expressed differs between cancers and normal breast, with the two additional domains AD1 and AD2 considered to be tumour associated. The aim of this study was to investigate expression of AD1 and AD2 in normal, benign and malignant breast tissue to determine their relationship to tumour characteristics and perform *in vitro* functional assays to investigate the role of AD1 in tumour cell invasion and growth.

Methods: Expression of AD1 and AD2 was related to hypoxanthine phosphoribosyltransferase 1 (HPRT1) as a housekeeping gene in breast tissue using reverse transcriptase qPCR ((RT)-qPCR) and results were related to clinicopathological features of the tumours. Constructs over-expressing an AD1-containing isoform (TNC-14/AD1/16) were transiently transfected into breast carcinoma cell lines (MCF-7, T-47D, ZR-75-1, MDA-MB-231 and GI-101) to assess the effect *in-vitro* on invasion and growth. Statistical analysis was performed using a non-parametric Mann-Whitney test for comparison of clinicopathological features with levels of TNC expression and Jonckheere-Terpstra trend analysis for association of expression with tumour grade.

Results: (RT)-qPCR detected AD1 and AD2 mRNA expression in 34.9% and 23.1% of 134 invasive breast carcinomas respectively. AD1 mRNA was localised by *in situ* hybridisation to tumour epithelial cells and more predominantly to myoepithelium around associated normal breast ducts. Although not tumour specific, AD1 and AD2 expression was significantly more frequent in carcinomas in younger women ($\leq$ 40yrs; P <0.001) and AD1 expression was also

associated with oestrogen receptor negative and grade 3 tumours ($P < 0.05$). AD1 was found to be incorporated into a tumour-specific isoform, not detected in normal tissues. Over-expression of the TNC-14/AD1/16 isoform significantly enhanced tumour cell invasion ($P < 0.01$) and growth ($P < 0.01$) over base levels.

Conclusions: Together these data suggest a highly significant association between AD containing tenascin-C isoforms and breast cancers in younger women (≤ 40yrs), which may have important functional significance *in vivo*.

Introduction

The role of the stromal microenvironment in modulating breast cancer behaviour is well established [1, 2]. A major modulatory component of the stromal environment is the extracellular matrix (ECM) and changes in ECM composition may therefore be expected to be a key factor in determining tumour behaviour. A consistent feature of the stroma around many breast carcinomas is up-regulation of the ECM glycoprotein tenascin-C [3-5].

Tenascin-C (TNC) is a complex multifunctional protein that can influence cell behaviour directly and indirectly [6, 7]. It has been shown to promote cell migration [8], inhibit focal adhesion formation [9], induce cell proliferation [6] and in some cases act as a cell survival factor [10]. TNC promotes angiogenesis [11] and can induce expression of Matrix Metalloproteinases (MMP) [12], which themselves have been implicated in promoting tumour growth and invasion [13].

Structurally TNC comprises a linear arrangement of domains, with a cysteine rich N-terminus followed by 14.5 EGF-like repeats, a region of fibronectin type III (FNIII)-like repeats and a fibrinogen-like domain at the C-terminus (Figure 1) [14]. The structure and size of TNC varies as a result of alternative splicing of domains within the FNIII repeat domain; exons 10-16 (domains A1-A4, B, C and D) can undergo alternative splicing either singly or in combination. A number of biologically active sites have been mapped to the FNIII repeat domain including recognition sites for cell surface receptors such as integrins [15], cell adhesion molecules of the immunoglobulin superfamily [16], and annexin II [17] as well as sites susceptible to proteolytic cleavage by MMP [18]. Thus inclusion or exclusion of different domains in this region can generate considerable functional diversity and up to 22 human splice variants have been identified by RT-PCR analysis [19].

Changes in the profile of TNC isoforms expressed in tumours compared to normal tissue have been described. A switch from the small or truncated form of TNC, which lacks exons 10-16, to predominant expression of the large, full-length variant of TNC has been demonstrated in breast, lung and colorectal carcinoma [20-22]. In glioblastoma, up-regulation of an isoform containing exon 15 (domain C), which is rarely detected in normal tissues or malignant epithelium, has been demonstrated [23]. We previously identified induction of two intermediate-sized TNC isoforms in breast cancers [24] and showed that these isoforms can enhance tumour cell invasion [25] and a similar change was demonstrated in ovarian carcinomas [26]. Although the precise significance of altered TNC isoform expression is not well understood, it seems likely that different isoforms have distinct biological function, a concept supported in recent studies [25, 27].

Two less well studied repeat regions have been identified in human, mouse and avian TNC, termed Additional Domain (AD) 1 and AD2, inserted between domains B and C [28-30]. Transcripts encoding AD1 have been detected in a variety of cancers [31] whilst inclusion of AD2 has so far been described only in oral squamous carcinomas [28]. Expression of both repeats has been detected in embryonic avian tissue. Interestingly, *in-situ* hybridisation demonstrated that TNC-AD1 mRNA is abundant at sites of cell motility and branching morphogenesis suggesting that inclusion of this repeat may contribute to the migration-promoting effect of TNC [31].

The potential to exploit tumour-specific alternatively spliced isoforms as therapeutic targets has already been demonstrated [32]. Thus the aims of this study were to establish whether TNC-AD1 and/or AD2 domains represent tumour-associated isoforms in breast cancer; to determine whether expression of these isoforms correlates with clinicopathological features and tumour subtypes identified by immunohistochemical profiling [33, 34]; and to use *in vitro* studies to investigate the functional significance of a TNC isoform containing the AD1

domain to tumour cell behaviour. The results of this study show that TNC-AD domains are significantly associated with breast cancers in younger women (< 40 years), high grade and ER negative status. Moreover, *in-vitro* cell culture models show that in some cell backgrounds AD1 containing isoforms promote tumour cell invasion and growth at a higher level than that seen with vector controls and other TNC isoforms.

Materials and methods

Tissues and cells

Fresh and/or formalin fixed tissue was obtained from patients undergoing breast surgery in accordance with Ethics Approval from Leicestershire LREC (06/Q2502/70) and North East London LREC (05/Q0403/199). Since all samples were anonymised there was no requirement from either REC for informed consent. For isolation of normal cells, tissue was obtained from women undergoing breast reduction surgery who gave informed consent (LNRREC 7054). 204 samples were analysed from 155 carcinomas selected based on age (68 infiltrating ductal carcinomas (IDC) from women ≤40 yrs, 62 IDC from women >40 yrs, 25 infiltrating lobular carcinomas (ILC) from women >40 yrs), 2 cases of ductal carcinoma *in-situ* (DCIS), 14 benign breast lesions including fibroadenoma and fibrocystic change and 33 normal breast samples from reduction mammoplasty procedures. Tissue was either snap-frozen in liquid nitrogen or routinely processed and paraffin embedded following formalin fixation. In selected normal samples, tissue was processed and enzymatically digested to yield single cell populations and myoepithelial, luminal epithelial and fibroblast cells were isolated and characterised as described previously [35].

Breast cell lines (MCF-7, ZR-75-1, T47D, Hs578T, MDA-MB-231, MDA-MB-468, MDA-MB-436, HBL-100 and MCF-10A) were obtained from American Type Culture Collection. Most cell lines were maintained in DMEM plus 2mM L-Glutamine and 10% Foetal Bovine Serum (FBS). ZR-75-1 and MDA-MB-436 were maintained in RPMI supplemented with 10% FBS**.** MCF-10A were maintained in 1:1 mixture of Ham's F12 (Invitrogen) and DMEM, 2mM glutamine supplemented with 5% heat inactivated horse serum, insulin (10μg/ml), hydrocortisone (0.5μg/ml) and epidermal growth factor (20ng/ml) (all Sigma).

Transfection of breast carcinoma cell lines

Breast cancer cell lines MCF-7, T-47D, ZR-75-1, MDA-MB-231 and GI-101 were transiently transfected with a novel TNC-14/AD1/16 construct, generated by PCR ligation using previously generated plasmids (TNC-S, TNC-L, TNC-9/14/16,) [25] and a Vector control (empty pCMV Script vector) and FuGene HD transfection reagent (Roche, UK) according to the manufacturer's protocol. For endogenously TNC expressing cell lines, co-transfection with the pmaxGFP vector (Lonza, UK) was also performed to assess transfection efficiency. TNC expression was confirmed using immunohistochemistry for TNC-null cell lines. For all cell lines, expression was also confirmed by reverse transcriptase qPCR ((RT)-qPCR). Transfection efficiencies for each isoform were confirmed by estimating the proportion of cells that were either immunostained positive or were expressing GFP.

RNA isolation and generation of cDNA

RNA was isolated from frozen tissue for 64 tumour samples, all benign and normal samples and 1 x 10^5 cells from each breast cell line using Tri-reagent (Sigma). 70 tumour tissue samples were available from a previous study in which mRNA was isolated using oligo-dT-linked Dynabeads® (Dynal, UK). 1μg of total RNA or all bead-isolated mRNA was reverse transcribed at 42^{o}C for 1 hour using Expand-RT (Boehringer Mannheim) as described previously [24].

Quantitative Polymerase Chain Reaction

Due to limiting amounts of tissue, a nested qPCR approach was devised to amplify TNC exons from frozen tissues. First round PCR comprised 20 cycles of amplification with primers located in domain 4 (exon 8) and domain 7 (exon 18), to amplify the entire

alternative spliced region of TNC (TNC 8/18). Taqman real-time PCR (Applied Biosystems, Foster, CA) was then applied to survey different exons. Inventoried assays were available for the Tenascin-C invariant exon 17/18 boundary (Applied Biosystems Taqman Assay, Hs01115654_m1) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Applied Biosystems Taqman Assay, Hs99999909_m1) as a housekeeping gene. Primers and probes were developed in house for AD1 and AD2 (Table 1). For the inventoried Taqman assays, 4 µl of cDNA (diluted 1:10) was analysed in a reaction containing 0.5 µl of probe, 0.5 µl UP H_2O and 5 µl of 2 x Taqman Fast PCR mastermix. For the AD1 and AD2 assays, 3.6 µl of cDNA (diluted 1:10) was analysed in a reaction containing 0.2 µl of probe, 0.6 µl of each primer and 5 µl of 2 x Taqman Fast PCR mastermix.

For all breast cell lines and a subset of the carcinomas (11 carcinomas ≤40 years and 11 carcinomas > 40 years) AD1, AD2 and exon 17/18 were also assayed directly to enable evaluation of AD1 and AD2 expression relative to total TNC expression (exon 17/18). All qPCR analysis was performed using the Step-One qPCR software from Applied Biosystems. The number of cycles necessary to produce a product above background (Ct value) was recorded and, after normalisation to the Ct value for HPRT1, the relative expression was determined with the formula: relative expression = $2^{-(\Delta\Delta Ct)}$

Determination of TNC transcript expression

The number of TNC transcripts was calculated using a standard curve generated from nested isoform-specific 8/18 PCR products. This was then used to calculate the number of molecules in a known concentration of sample. A $\log_2$ value was then produced from the mean Ct value and normalised against the mean Ct values for the endogenous controls.

Sequencing

AD1 amplicons were analysed by DNA sequencing. Products were sequenced from AD1-F and AD1-R primers respectively (Table 1) using 1μl Big Dye Terminator reactions (ABI) and analysed on an ABI Prism 377 DNA sequencer. Sequence profiles were analyzed using the Chromas software (version 2.3, Technelysium Pty, Ltd).

Immunohistochemistry

Immunohistochemistry for Cytokeratin (CK) 14 (Sigma), CK5/6 (Sigma) and P-Cadherin (BD Biosciences), was performed on 4μm FFPE serial sections from a subset of 96 breast carcinomas. A standard Avidin Biotin Complex technique was employed with citrate buffer microwave antigen retrieval for P-Cadherin. Normal breast tissue was used as a positive control for all antibodies. Negative controls involved omission of the primary antibody. Sections were scored positive for CK14, CK5/6 or P-Cadherin if more than 10% of the tumour cells were stained. Oestrogen Receptor (ER) and Progesterone Receptor (PgR) were examined in 138 tumour cases and for HER2 in 134 cases [36]. All interpretation was carried out by RAW and JLJ.

Western blotting

Levels of cellular and secreted TNC isoforms were determined by western blotting of transfected cell lysates and serum free culture media (CM) respectively. Cells were transiently transfected and incubated for 24 hours, serum-free media was added and the CM collected a further 48 hours after transfection. Protein concentrations were quantified on a Lambda 25 UV/VIS spectrophotometer at 750 nm using the BSA protein assay, and equal amounts of protein were loaded onto 6% SDS-PAGE gels and transferred to Hybond ECL

nitrocellulose membrane (Amersham Biosciences, UK). Membranes were blocked in Tris-buffered saline, 5% milk and 1% Tween for 1 hour and probed for 1 hour with a rabbit polyclonal TNC antibody (clone H300, recognising all forms of TNC; Santa-Cruz, USA). A secondary antibody, donkey anti-rabbit HRP-linked IgG, 1:2000 (Amersham Biosciences, UK), was added for 1 hour and blots were detected using an enhanced chemiluminescence detection kit (Amersham Biosciences, UK).

In-situ localisation of AD1 containing mRNA

Single-stranded sense and antisense AD1 and β-actin control DNA probes were synthesised using asymmetric PCR [24] with specific primers (Table 1) and incorporation of digoxigenin-11-dUTP. *In-situ* hybridisation was carried out on 4µm de-waxed rehydrated tissue sections as described previously [24]. The optimum probe concentration (200-500 ng/ml) was titrated to eliminate background staining, whilst retaining good signal strength. Negative controls were using sense probes, RNase pre-treatment of tissue sections and omission of the probe in the hybridisation protocol.

Analysis of tumour cell invasion

Measurement of tumour cell invasion was based on modified Boyden Chamber assays using the Fluoroblok tumour cell invasion system as described previously [37]. To measure the direct effect of TNC isoform expression on tumour cell invasion, transiently transfected cells were placed in the upper chamber of the assay and DMEM containing 10% (v/v) FCS added to the lower chamber as a chemotactic stimulus except for MDA-MB-231 where DMEM containing 1% (v/v) FCS was used. Assays were monitored in real time up to 48 hours with

readings every 2 hours and for a total of 6 replicates representing three separate transfections. Relative fluorescence to time 0 was used as a measure of invasion for each time point,

Analysis of tumour cell growth

The direct effects of TNC isoform expression on tumour cell growth were assessed using DAPI-staining of cell nuclei as described previously [38]. Tumour cells were transfected, and cultured for 48 hrs in DMEM containing 10% (v/v) FCS. Cells were washed with PBS, fixed in cold acetone/methanol (1:1) for 2 min and washed twice with PBS. Cells were then stained using DAPI (1:20000) in PBS for 3 min and washed twice with PBS. Cell nuclei were examined using a fluorescence microscope and counted using an in-house software program provided by Dr AE Sayan (University of Leicester, UK) [38]. Each experiment was performed for a total of 9 replicates representing 3 separate transfections.

Statistical Analysis

Statistical analysis was performed using SPSS 16. Relationships between factors were measured using the non-parametric Mann-Whitney test, except for grade where Jonckheere-Terpstra trend analysis was applied. All tests were two-sided and a P value of <0.05 was considered significant. For functional assays, two-way ANOVA was performed.

Results

Expression of TNC-AD1 and TNC-AD2 in breast cell lines and isolated cell populations

Nine breast cell lines and isolated normal breast myoepithelial and fibroblast cells were screened for expression of TNC 17/18 (an invariant exon boundary, present in all TNC splice variants), AD1 and AD2 by real-time quantitative PCR. TNC 17/18, AD1 and AD2 were absent from ER positive MCF-7, ZR-75-1, and T-47D cells (Table 2). Of the AD1 positive cell lines, MDA-MB-436, HBL-100 and primary normal myoepithelial cells expressed the highest levels (>400.000 molecules per 1µg RNA). All AD1 positive cell lines and isolated breast cell populations also expressed AD2 but at lower levels. When AD1 and AD2 expression was related to levels of total TNC expression, HS578T, HBL100 and primary myoepithelial cells showed the highest percentages of AD1 (> 25% total TNC) and HS578T and MCF10A showed highest AD2 (>5% total TNC).

Expression of TNC-AD1 and AD2 in breast tissues

RNA was successfully isolated from 134 carcinomas, all 14 benign and 33 normal breast tissue samples, demonstrated by detection of GAPDH by manual PCR. All samples were then analysed by (RT)-qPCR for expression of the invariant TNC exon 17/18 boundary (total TNC), AD1 and AD2. Total TNC was detected in all samples apart from 2 of the carcinomas. AD1 expression was demonstrated in 42 (31%) carcinomas, 5 benign breast tissues and 12 normal breast tissues (8 of which were from women aged <40 years). TNC-AD2 was less frequent than TNC-AD1, being detected in 31 (23.1%) carcinomas, 4 benign and 4 normal breast samples. 19 carcinomas, 1 benign sample and 1 normal sample expressed both AD1 and AD2.

TNC-AD1 and AD2 expression in relation to clinicopathological features of carcinomas

When quantitative data was compared to clinicopathological features of the carcinomas, expression of AD1 was significantly associated with grade 3 tumours, ER negative status and with younger patient age (≤40yrs) (Table 3). AD2 expression was also significantly associated with younger patient age (Table 3). When the data were stratified by presence of exons only, 19 carcinomas were positive for both AD1 and AD2, and 16 of these were from women ≤40yrs in age ($P < 0.001$, Figure 2) and the highest frequency of TNC-AD1 and AD2 positive tumours was seen in the 31-35 yr age category. Neither AD1 nor AD2 showed any association with lymph node status.

For 22 of the carcinomas (11 ≤40 years, 11 > 40 years) AD1, AD2 and exon 17/18 were assayed directly. The total number of TNC molecules and transcripts containing AD1 was greater in carcinomas from younger women (Table 4). It was not possible to statistically analyse AD2 expression since only 4 samples were positive.

TNC-AD1 and AD2 expression was also analysed in relation to the tumour subtype, as determined by immunohistochemical profiling [33, 34]. Nielsen *et al.* [34] considered that lack of ER and HER2 and presence of Epidermal Growth Factor Receptor (EGFR/HER1) and cytokeratin 5/6 could identify basal-like carcinomas. Carey *et al.* [33] defined basal-like as (ER-, PR-, Her2-, CK5/6 and/or CK14+). There has been debate as to whether "triple negative" (i.e. ER-, PR-, HER2-) cancers are the same as basal-like tumours [39]. Rakha *et al*. [40] have recently proposed that cytokeratins 5/6 and/or 14 can be used to define basal-like carcinomas irrespective of the expression of the other markers. We therefore chose to examine a range of putative basal markers (ER-, PR-, HER2-, CK5/6 and 14, and P-cadherin [41]) and compared the relationship of these with AD1and AD2 expression.

Immunohistochemical profiling and TNC-AD1/AD2 status was known in 132 cases (59 ≤40 yrs and 73 >40 yrs) for "basal phenotype 1" defined as triple negative tumours, and in 73 cases (44 ≤40 yrs and 29 >40 yrs) for "basal phenotype 2" defined as cytokeratin 5/6 and/or 14 positive tumours. There was no significant relationship between TNC-AD1/2 statuses and either basal subtype (Table 3). There was also no relationship between TNC-AD1/2 status and either HER-2 or P-Cadherin (data not shown).

The AD1 domain is part of novel intermediate-sized TNC isoforms

Sequencing was carried out on 2 tumour samples that showed expression of two different sized AD1 containing isoforms (Figure 3a), the largest of which was tumour specific. Blast sequence analysis [42] confirmed that AD1 was located within one of two different intermediate sized TNC transcripts between exons 9/14 and either exons 15/16 or exon 16 alone (Figure 3b; sequence of AD1 [GenBank:EU295718]). Neither isoform contained AD2. Analysis of normal breast cell populations isolated from reduction mammoplasties demonstrated a single isoform TNC-14/AD1/16 in myoepithelial cell populations from 5 separate donors and in 5 of 12 fibroblast populations.

TNC-AD1 is derived largely from epithelial cells

In-situ hybridisation was carried out on 8 breast carcinomas with known TNC-AD1 status using TNC-AD1 probes and β-actin as a control (Figure 4). Specificity of signal was established using sense probes, which were consistently negative. Two carcinomas, which were negative for TNC-AD1 by (RT)-qPCR, were also negative by *in-situ* hybridisation. In six TNC-AD1 positive breast carcinomas the TNC-AD1 antisense probe localised to tumour

cells and not to stromal cells (Figure 4a), and in the associated myoepithelial cells around large histologically normal ducts there was a lower level of expression detected (Figure 4b).

Direct effects of TNC isoforms on invasion and growth

Although the exon 15-containing isoform (TNC-14/AD1/15/16) was successfully cloned, it was not expressed at the protein level (as determined by Western blotting – see Additional file 1) and therefore functional studies focused on the TNC-14/AD1/16 isoform. Transient transfection was used to determine the direct effects of TNC isoforms (TNC-S, TNC-9/14/16, TNC-14/AD1/16) on invasion in the 3 TNC null cell lines (MCF-7, T-47D, ZR-75-1) and two cell lines with endogenous TNC expression (MDA-MB-231 and GI-101) (Figure 5). All cell lines transfected with TNC-S showed no significant increase in invasion compared to vector controls. TNC-14/AD1/16 transfected MCF-7, T-47D, MDA-MB-231 and GI-101 showed increased invasion over vector controls, which became significant with increasing time (Table 5). No significant increase in invasion was evident in ZR-75-1. TNC-9/14/16 transfected MCF-7 and GI-101 cells also showed increased invasion over vector controls with time. No significant increase in invasion was observed in T-47D, ZR-75-1 or MDA-MB-231 (Figure 5 and Table 5). A similar trend in invasion was observed when compared to TNC-S; however, significance appeared at a later time point (Table 5). In MCF-7 cells we also compared mean invasion induced by TNC-14/AD1/16 and TNC-L (this isoform contains all of the exons in the alternatively spliced region, with the exception of AD1 and AD2). TNC-14/AD1/16 significantly enhanced tumour cell invasion over cells expressing TNC-L ($P < 0.001$) (Figure 5a(v)). (RT)-qPCR analysis of transfected TNC expression showed equivalent levels between constructs in all cell lines, except in MCF-7 cells, which showed TNC-S to be expressed at a higher level (Figure 5b).

Transient transfection was also used to determine the direct effects of TNC isoforms (TNC-S, TNC-9/14/16, TNC-14/AD1/16) on cell growth in MCF-7, T-47D and MDA-MB-231 cells. A significant increase in cell number was observed 48 hrs post transfection in both MCF-7 and MDA-MB-231 cells over-expressing TNC-9/14/16 and TNC-14/AD1/16 ($P < 0.01$ and < 0.001 respectively for MCF-7 and $P < 0.001$ for both in MDA-MB-231) compared to the vector control. Furthermore, a significant increase was observed in MCF-7 cells over-expressing TNC-S ($P < 0.01$). No significant increase was observed in MDA-MB-231 cells over-expressing TNC-S. However, MDA-MB-231 cells over-expressing TNC-9/14/16 and TNC-14/AD1/16 both showed a significant increase in cell numbers compared to TNC-S ($P < 0.001$ for both). No significant differences were found between isoforms and vector controls for T-47D (Figure 6).

Discussion

The tenascin-C isoforms containing AD1 and/or AD2 domains have been suggested to be tumour specific [28, 31], and TNC-AD1 may contribute to a motility-promoting environment [30]. This study has shown that expression of only one AD1 containing variant (TNC-14/AD1/15/16) was cancer specific, but higher mRNA levels of all AD1 and AD2 variants were associated with high grade, ER negative breast cancers, and cancers arising in younger women. Detection of AD2-containing isoforms was less frequent and at lower levels; therefore, we focused on determining the functional significance of AD1 variants and showed that the TNC-14/AD1/16 isoform promoted invasion and growth.

This study analysed a series of breast cancer cell lines and also isolated cell populations from normal breast ($n > 5$). (RT)-qPCR demonstrated expression of TNC-AD1 and AD2 isoforms in all TNC positive lines, which were also oestrogen receptor (ER) negative and characterised

by aggressive behaviour [43-45]. Analysis of isolated cell populations from normal breast showed that the predominant source of AD1 and AD2 expression is the myoepithelium. Isolated stromal fibroblasts, the source of most TNC isoforms [24], showed only low level expression that was not detected for AD1 by *in-situ* hybridisation. The cell line HBL-100 also showed a high level of expression and this exhibits myoepithelial cell characteristics [46]. AD1 was not detectable in normal tissues by *in-situ* hybridisation; but was detected at a low level in isolated enriched populations of a single normal cell type. For the breast cancers the cells expressing AD1 were predominantly malignant epithelial cells, rather than fibroblasts. Sequencing of the two AD1 variants identified in cancers, with AD1 incorporated into an isoform containing exons 15 plus 16 or 16 alone, are distinct from those described by Mighell *et al* [28]. In their study of oral tissues, AD1-containing isoforms were identified in normal and reactive lesions as well as tumour tissue, with TNC-AD2 expression to be rare but tumour-specific. In this study the TNC-14/AD1/15/16 variant was cancer specific as this was not detected in any of the normal tissues or isolated cell populations.

We demonstrated that the TNC-14/AD1/16 isoform significantly increased breast cancer cell invasion and growth. The largest TNC isoform (TNC-L) has been frequently associated with an invasive phenotype [6, 9, 27], as have two other prominent isoforms containing exons 16 and 14/16 [24, 25]. However, the TNC-14/AD1/16 isoform showed a much greater effect on tumour cell invasion than TNC-L in MCF-7 cells, suggesting that these intermediate sized isoforms may be most biologically relevant. TNC-14/AD1/16 also produced a greater effect than the TNC-9/14/16 isoform but this was only significant for MCF-7 and T-47D. The fully truncated TNC isoform (TNC-S) showed no significant effect on tumour cell invasion, consistent with previous reports [25].

TNC-14/AD1/16 was also shown to increase cell growth in MCF-7 cells at a similar level to TNC-9/14/16, supporting our previous data [26]. However, in cells that endogenously

express TNC (i.e. MDA-MB-231), transfection with TNC-14/AD1/16 had a much greater effect on cell growth. The precise mechanisms leading to increased cell invasion and growth in the breast are unclear. Ruiz *et al.* [47] and Lange *et al.* [48] have associated triggering of glioma cell migration with a simultaneous mechanism of competitive inhibition of syndecan-4 binding of fibronectin and signalling by lysophosphatidic acid (LPA) and platelet-derived growth factor (PDGF) (reviewed in [49]). Furthermore, TNC has been shown to be susceptible to degradation by MMPs [50, 51], which can reveal cryptic sites present in the TNC molecule [52], of these AD1 could be a potential site. Further work using peptide fragments containing AD1 could help elucidate these functions.

High expression of either AD1 or AD2 containing mRNA in breast cancers was significantly associated with young patient age (≤40 years). AD1 expression was also associated with lack of oestrogen receptor and grade 3, both of which are features of cancers in younger women. These results correlate with findings by Helleman *et al.* [53], who suggested that high TNC expression could be an indirect marker for a defective ER pathway due to an inverse correlation between TNC mRNA and ER protein expression. Breast carcinomas in young women exhibit a particularly aggressive phenotype compared to tumours arising in post-menopausal women [54-56]. This may in part be attributed to the greater frequency of high-grade tumours in young women [57]. However, there is evidence that tumours in young women are biologically distinct, being associated with poorer survival independent of tumour grade and stage [58] and showing a higher frequency of loss of heterozygosity (LOH) compared to grade- and stage-matched post-menopausal cancers [59]. The association of AD1 and AD2 expression with younger age supports the hypothesis that these tumours are biologically distinct.

Over recent years there has been increasing recognition of a subgroup of breast carcinomas characterised by high levels of expression of genes and proteins normally associated with the

myoepithelial or basal cell population of the breast [33, 34, 36, 60]. This basal tumour subtype exhibits a poorer prognosis than other subgroups [60], occurs with high frequency in BRCA1-mutated tumours [36] and more frequently in younger women [60]. To address whether the TNC-AD1 (myoepithelial-associated) and TNC-AD2 isoforms were associated with basal features, we compared TNC-AD1 status to expression of putative basal markers [33, 34]. There was no association of TNC-AD1 expression with either the triple negative or cytokeratins 5/6 and/or 14 positive subtypes, or with any of the other putative basal markers. This may be due to relatively small numbers of "basal" cases available for analysis, but a larger study was precluded by the need for fresh tissue for analysis.

Conclusions

In conclusion this study has shown a highly significant association between expression of TNC-AD1 and TNC-AD2 and carcinomas arising in young women (≤40 years), and TNC-AD1 is sometimes incorporated into a novel tumour-associated TNC isoform not detected in normal tissues. The association of TNC-AD1 expression with tumours arising in young women and also with high grade and ER negative tumours make it a plausible target for development of novel therapies. Functional studies showed that the TNC-14/AD1/16 isoform can significantly increase breast cancer cell invasion and growth, to a greater extent than the previously described tumour associated TNC-9/14/16 isoform. This raises interesting questions regarding the functional significance of TNC isoforms containing the AD domains.

Abbreviations

AD: additional domains; BSA: bovine serum albumin; CM: conditioning medium; ECM: extracellular matrix; EGF: epidermal growth factor; ER: oestrogen receptor; DMEM:

Dulbecco's modified Eagle's medium; FBS: foetal bovine serum; FN III: fibronectin type III-like repeats; HPRT1: hypoxanthine phosphoribosyltransferase 1; LOH: loss of heterozygosity; MMP: matrix metalloproteinase; PCR: polymerase chain reaction; PR: progesterone receptor; RT: reverse transcriptase; RT-PCR: reverse transcriptase polymerase chain reaction; TIMP: tissue inhibitor of matrix metalloproteinases; TNC: tenascin-C; TNC-9/16: tenascin-C with only exon 16 of variable region; TNC-9/14/16: tenascin-C with only exons 14 and 16 of variable region; TNC-14/AD1/16: tenascin-C with only exons 14, AD1 and 16 of variable region; TNC-L: tenascin-C largest splice variant; TNC-S: tenascin-C fully truncated splice variant.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JHP, JAS and DSG created the TNC isoform expression vectors. DSG carried out (RT)-qPCR assays, invasion assays, cell growth assays and blotting. RAH carried out extraction of RNA from breast tissue, nested RT-PCR assays and extracted RNA from isolated myoepithelial cell and fibroblast populations. KTM and SH performed immunostaining of tumour tissue, which was reviewed by JLJ and RAW. SL isolated primary cells and RNA from breast reductions. JHP, JLJ, JAS, RAH, RAW and DSG conceived the study, participated in its design and co-ordination and helped to draft the manuscript. JHP, DSG and JAS performed statistical analysis. All authors read and approved the final manuscript.

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Figure 1: Schematic representation of Tenascin-C. Protein structure illustrating the N-terminal Tenascin Assembly domain, the Epidermal Growth Factor (EGF)-like repeat region, the Fibronectin type III (FNIII)-like region and the C-terminal Fibrinogen-like domain. Exon organisation is shown below (adapted from [25]). Note that first exon of TNC is not translated and is shown in grey; therefore, exon number 1 in this figure is the first coding exon.

Figure 2: Distribution of AD positive and AD negative breast carcinomas in relation to patient age. Number of carcinomas positive or negative for both AD1 and AD2. The box contains cases within the lower and upper quartiles and the line indicates the median age. The whiskers connect the youngest and oldest patients that are not outliers, whilst o indicates outliers and * indicates extreme values, defined as more than 3 box-lengths from the box.

Figure 3: Identification of two distinct AD1-containing TNC isoforms. (a) RT-PCR analysis in two breast cancers using T8-F and AD1-R primers (Tracks 1 and 2) and AD1-F and T16-R primers (Tracks 3 and 4). B identifies blank lane. Two alternatively spliced isoforms identified by sequencing are shown schematically in **(b)**.

Figure 4: *In-situ* localisation of TNC-AD1 to tumour cells and normal myoepithelial cells. (a) An example of infiltrating ductal carcinoma positive for TNC-AD1 by RT-PCR. (i) H&E, (ii) ISH using antisense probe to TNC-AD1 domain, demonstrating signal in tumour cells, (iii) ISH using TNC-AD1 sense probe as a negative control. **(b)** A normal breast duct with adjacent tumour cells within a lymphovascular space. (i) H&E, (ii) ISH with antisense

probe to TNC-AD1 showing signal in scattered myoepithelial cells of normal ducts as well as localised to tumour cells. (iii) ISH with TNC-AD1 sense probe as a negative control.

Figure 5: Direct effects of TNC isoform expression on tumour cell invasion. (a) Monitoring tumour cell invasion in real-time using a modified Boyden chamber assay (i) MCF-7, (ii) T-47D, (iii) MDA-MB-231 and (iv) GI-101 cell lines transfected with 3 different TNC isoform constructs and the vector control. (v) Invasion of MCF-7 cells transfected with TNC-L and TNC-14/AD1/16 constructs. In all cases, data points represent the mean of six replicates representing three separate transfections with error bars shown. **(b)** (RT)-qPCR of total TNC transcripts (exons 17/18) from cells transfected with TNC constructs.

Figure 6: Direct effect of TNC isoform expression on tumour cell growth. (a) Growth of MCF-7, MDA-MB-231 and T-47D cell lines transfected with three different TNC constructs and a Vector control. Bars represent the mean relative cell count compared to 0 hrs, with each assay performed three times in triplicate and error bars shown. **(b)** Images of cells transfected with three different TNC constructs. Images were taken using a 4x magnification in exactly the same position in each well. ** $P < 0.01$, *** $P < 0.001$.

Table 1: Primer sequences for RT-PCR and asymmetric PCR

Primer	Sequence 5' – 3'
T8-F	CAATCCAGCGACCATCAACG

T18-R	CGTCCACAGTTACCATGGAG
T16-R	GTTGTCAACTTCCGGTTCGG
AD1-F	TGGTGGAGAACACTGGCTATGAC
AD1-R	GGGATCCCCAGCCAAGGT
AD1-FAM-MGB	CAGTGTGGCAGGAAC
AD2-F	GATCACCCCCATGAGACCAT
AD2-R	TGATGACAGAGCTGC GAGACA
AD2-FAM-MGB	TGCTGTCTGTGCCTGG
GAPDH-F	AGAACATCATCCCTGCCTC
GAPDH-R	GCCAAATTCGTTGTCATACC
Actin Anti-sense	TCATCACCATTGGCAATGAG
Actin Sense	CTAGAAGCATTTGCGGTGGA
AD1 Anti-sense	GAACCAAAGCCACAGTTGG
AD1 Sense	TAATGACAAAGGCAGTGAG

F: Forward primer; R: Reverse primer; Sense and anti-sense primers were used to generate single stranded DNA probes for *in-situ* hybridisation.

Table 2: Relative expression of tenascin-C AD1 and AD2 in cell lines and isolated normal breast cells

	Number of TNC transcripts (x10^3)			% of total TNC transcripts	
Cell line	**Total TNC**	**AD1**	**AD2**	**AD1**	**AD2**
ZR-75-1	0	0	0	0	0
T-47D	0	0	0	0	0

MCF-7	0	0	0	0	0
Hs578T	162.651	41.432	14.574	25.47	8.96
MDA-MB-231	1293.947	44.383	23.821	3.43	1.84
MDA-MB-436	8409.526	479.824	55.735	5.71	0.66
MDA-MB-468	984.727	89.74	28.784	9.11	2.92
GI-101	1529.834	143.854	8.433	9.40	0.55
HBL-100	15017.045	6671.261	483.212	44.42	3.22
MCF-10A	91.37	7.399	6.933	8.10	7.59
Primary MEC	2925.394	1863.143	142.39	63.69	4.87
Primary fibroblasts	5398.821	87.867	98.044	1.63	1.82

Percent expression of AD1 and AD2 relative to the invariant exon 17/18 boundary, as a marker of total tenascin-C expression, normalised to the HPRT1 control in cell lines and isolated normal breast cells. Primary MEC = Primary myoepithelial cells.

Table 3: Relationship between expression of total TNC, TNC-AD1 and AD2 and clinicopathological features of tumours

Clinicopathological feature		n	Total TNC		TNC-AD1		TNC-AD2	
			Mean Ct (95% CI)	*P*	Mean Ct (95% CI)	*P*	Mean Ct (95% CI)	*P*
Age	≤ 40 years	47	23.5 (21.8 - 25.3)	0.006	36.6 (35.4 - 37.7)	<0.001	37.7 (36.7 - 38.6)	0.001
	> 40 years	87	26.5 (25.7 - 27.3)		38.7 (38.1 - 39.3)		39.1 (38.5 - 39.7)	
Tumour type	IDC	109	25.4 (24.4 - 26.4)	0.554	37.8 (37.2 - 38.4)	0.079	38.7 (38.2 - 39.2)	0.760
	ILC	25	25.6 (24.7 - 26.5)		38.6 (37.1 - 40.0)		38.3 (36.5 - 39.9)	
Grade	I	14	27.6 (25.9 - 29.4)	0.109	39.1 (37.9 - 40.3)	0.017	39.8 (39.3 - 40.2)	0.066
	II	63	25.7 (24.6 - 26.7)		38.3 (37.4 - 39.1)		38.7 (37.9 - 39.5)	
	III	57	24.7 (23.2 - 26.1)		37.3 (36.4 - 38.2)		38.2 (37.4 - 39.0)	
Lymph node	Pos	63	25.4 (24.2 - 26.6)	0.802	37.9 (37.2 - 38.8)	0.978	38.8 (38.2 - 39.4)	0.900
	Neg	68	25.5 (24.4 - 26.7)		37.9 (37.1 - 38.8)		38.5 (37.7 - 39.3)	
	n/k	3						
ER	Pos	89	25.6 (24.6 - 26.5)	0.413	38.2 (37.6 - 38.9)	0.011	38.7 (38.1 - 39.3)	0.316
	Neg	31	24.1 (22.2 - 26.1)		36.7 (35.3 - 38.0)		38.1 (36.9 - 39.3)	
	n/k	14						
PR	Pos	79	25.4 (24.5 - 26.4)	0.431	37.9 (37.2 - 38.4)	0.441	38.8 (38.2 - 39.3)	0.641
	Neg	36	24.5 (22.6 - 26.4)		37.6 (36.4 - 38.7)		38.1 (36.8 - 39.5)	
	n/k	19						
Her-2	Pos	17	23.3 (20.5 - 26.2)	0.232	37.6 (35.9 - 39.3)	0.639	38.2 (36.6 - 39.8)	0.452
	Neg	81	25.1 (24.2 - 26.1)		37.8 (37.0 - 38.6)		38.7 (38.0 - 39.4)	
	n/k	36						
Basal 1	Pos	23	24.3 (22.2 - 26.4)	0.605	36.9 (35.3 - 38.6)	0.170	38.4 (37.2 - 39.7)	0.746
	Neg	109	25.4 (24.5 - 26.3)		38.0 (37.4 - 38.7)		38.6 (37.9 - 39.1)	
	n/k	2						
Basal 2	Pos	22	25.8 (23.1 – 28.4)	0.103	37.6 (35.9 – 39.4)	0.960	38.5 (37.3 – 39.6)	1.000
	Neg	51	23.2 (21.4 – 25.0)		37.6 (36.6 – 38.7)		38.5 (37.7 – 39.3)	
	n/k	61						

IDC: infiltrating ductal carcinoma; ILC: infiltrating lobular carcinoma; ER: oestrogen receptor; PR: progesterone receptor; LN: lymph node; +: positive; -: negative; n/k: not known; TNP: Triple negative phenotype; 95% CI: 95% confidence intervals.

Table 4: TNC transcript levels in carcinomas stratified by age

		Number of molecules			Mean		
	Sample	**Total TNC**	**TNC-AD1**	**TNC-AD2**	**Total TNC**	**TNC-AD1**	**TNC-AD2**
≤ 40 years	T1	1212265	52620	0	143696985	8944007	2027925
	T2	99024132	1567084	0	(*P* = 0.005)	(*P* = 0.030)	
	T3	2088668	150030	118774			
	T4	958188	58137	0			
	T5	4701400	0	0			
	T6	14962216	0	0			
	T7	11837854	197415	0			
	T8	339673	0	0			
	T9	192103000	17251715	11618505			
	T10	1100396396	79107073	10569893			
	T11	153043046	0	0			
> 40 years	T12	37859646	0	0	4677309	25914	2211
	T13	208821	37702	24325			
	T14	7992309	0	0			
	T15	66360	7451	0			
	T16	897614	0	0			
	T17	73244	0	0			
	T18	1206237	229110	0			
	T19	168882	9045	0			
	T20	276036	0	0			
	T21	6970	1746	0			
	T22	2694279	0	0			

Normalised expression of TNC molecules in carcinomas from women age ≤ 40 years and > 40 years. Mann-Whitney test were used to calculate P values.

Table 5: Analysis of cell invasion following transient transfection with TNC isoforms

Isoform	Time (hrs)	MCF-7	T-47D	MDA-MB-231	GI-101	ZR-75-1
TNC-S	12	ns	ns	ns	ns	Ns
	24	ns	ns	ns	ns	Ns
	36	ns	ns	ns	ns	Ns
	48	ns	ns	ns	ns	Ns
TNC-9/14/16	12	ns	ns	ns	ns	Ns
	24	ns	ns	ns	ns	Ns
	36	p = <0.05 (ns)	ns (ns)	ns (ns)	p = <0.05 (<0.05)	ns (ns)
	48	p = <0.001 (<0.05)	ns (ns)	ns (ns)	p = <0.001 (0.05)	ns (ns)
TNC-14/AD1/16	12	ns	ns	ns	ns	Ns
	24	p = <0.05 (ns)	p = <0.05 (ns)	p = <0.01 (ns)	ns (ns)	ns (ns)
	36	p = <0.001 (<0.001)	p = <0.001 (<0.001)	p = <0.001 (<0.01)	ns (ns)	ns (ns)
	48	p = <0.001 (<0.001)	p = <0.001 (<0.001)	p = <0.001 (<0.001)	p = <0.01 (ns)	ns (ns)

Significance at 12 hour intervals relative to vector only controls or TNC-S (in brackets). Ns: not significant.

Additional file 1: Supplementary Figure 1. Western blot analysis of TNC expression in transiently transfected MCF-7 cells. This demonstrated a single species of TNC present in the conditioning media and whole cell lysate. TNC-S is seen as a band at approximately 200 kDa, with slightly larger bands detected for TNC-B/D (exons 14/16) and TNC-B/AD1/D (exons 14/AD1/16), with TNC-B/AD1/D detected at approximately 270 kDa.

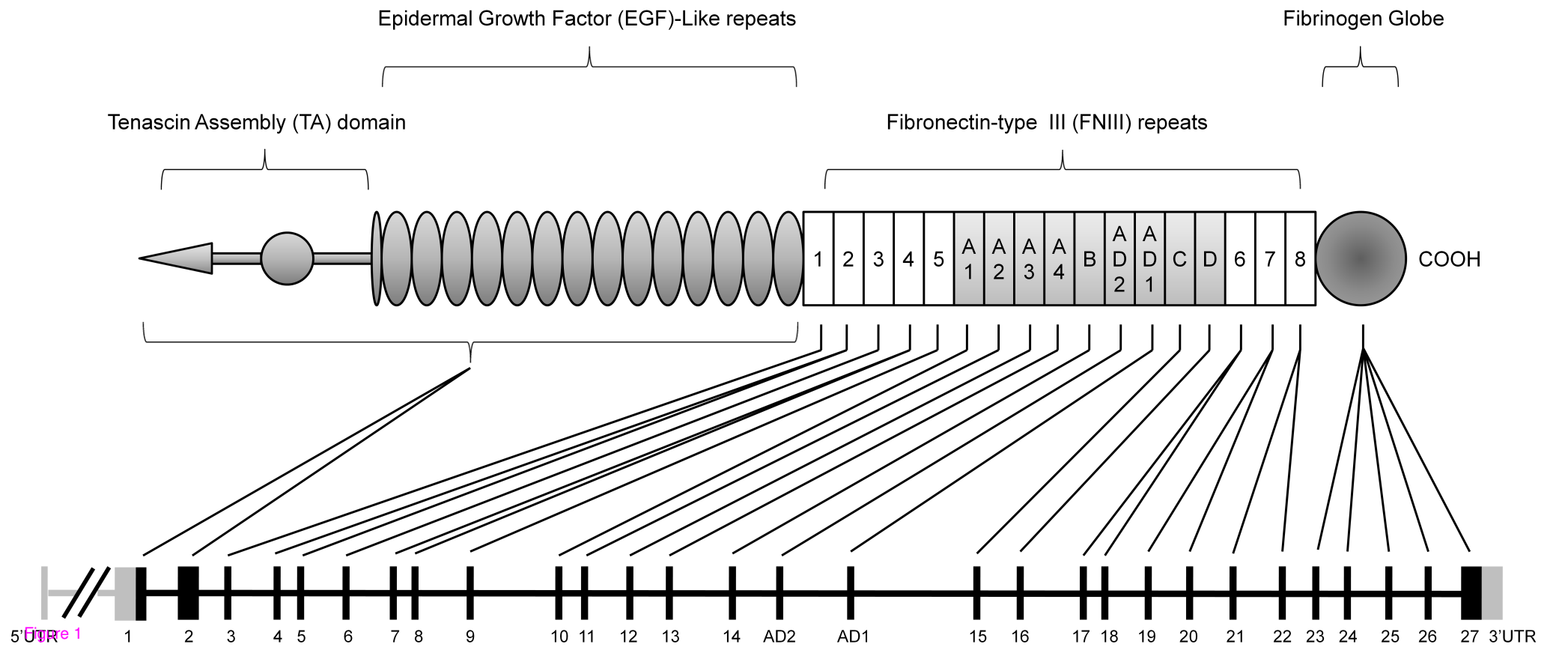

Epidermal Growth Factor (EGF)-Like repeats
Fibrinogen Globe
Tenascin Assembly (TA) domain
Fibronectin-type III (FNIII) repeats
1
2
3
4
5
A 1
A 2
A 3
A 4
B
A D 2
A D 1
C
D
6
7
8
COOH
5'UTR
1
2
3
4
5
6
7
8
9
10
11
12
13
14
AD2
AD1
15
16
17
18
19
20
21
22
23
24
25
26
27
3'UTR

Figure 1

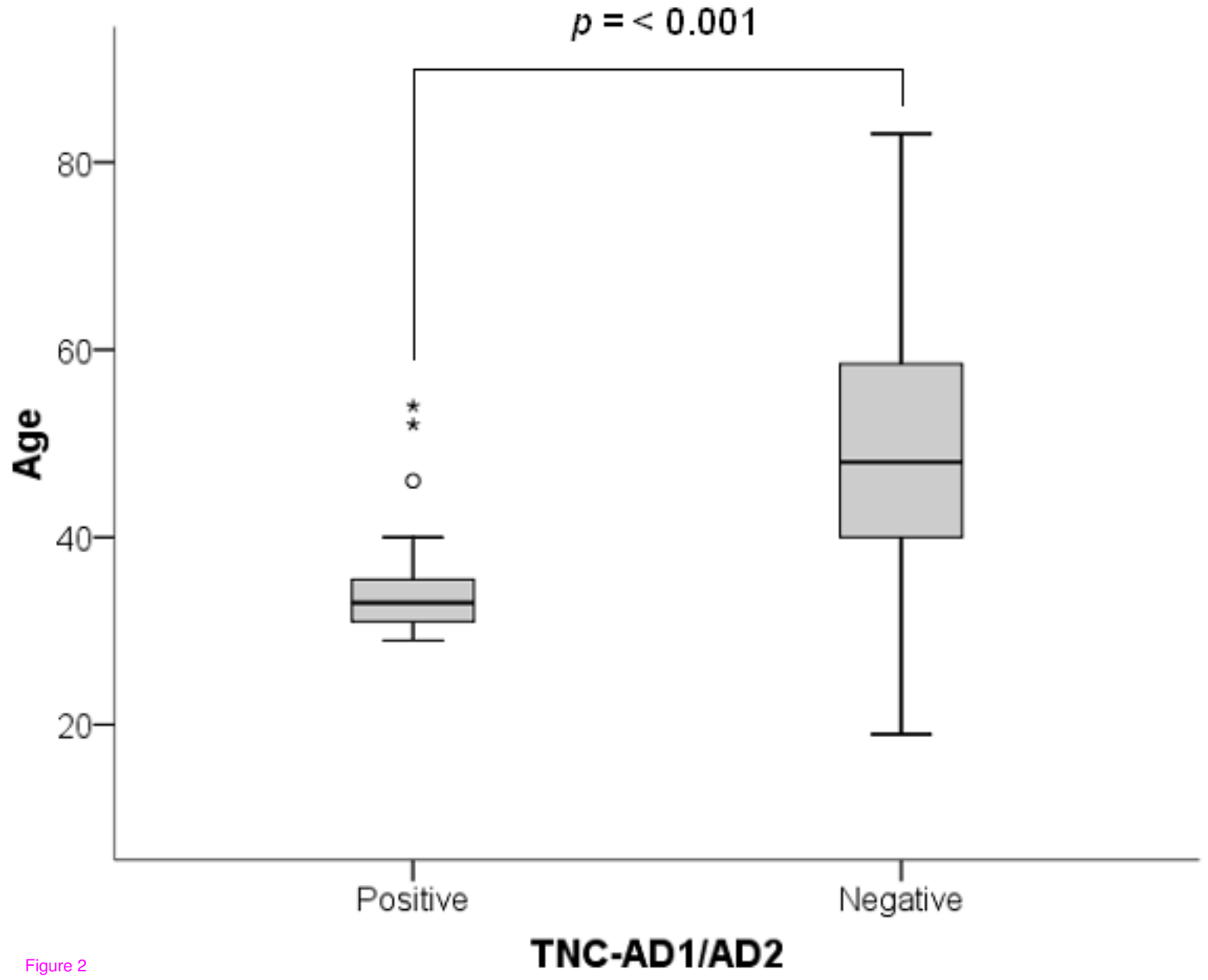


Figure 2

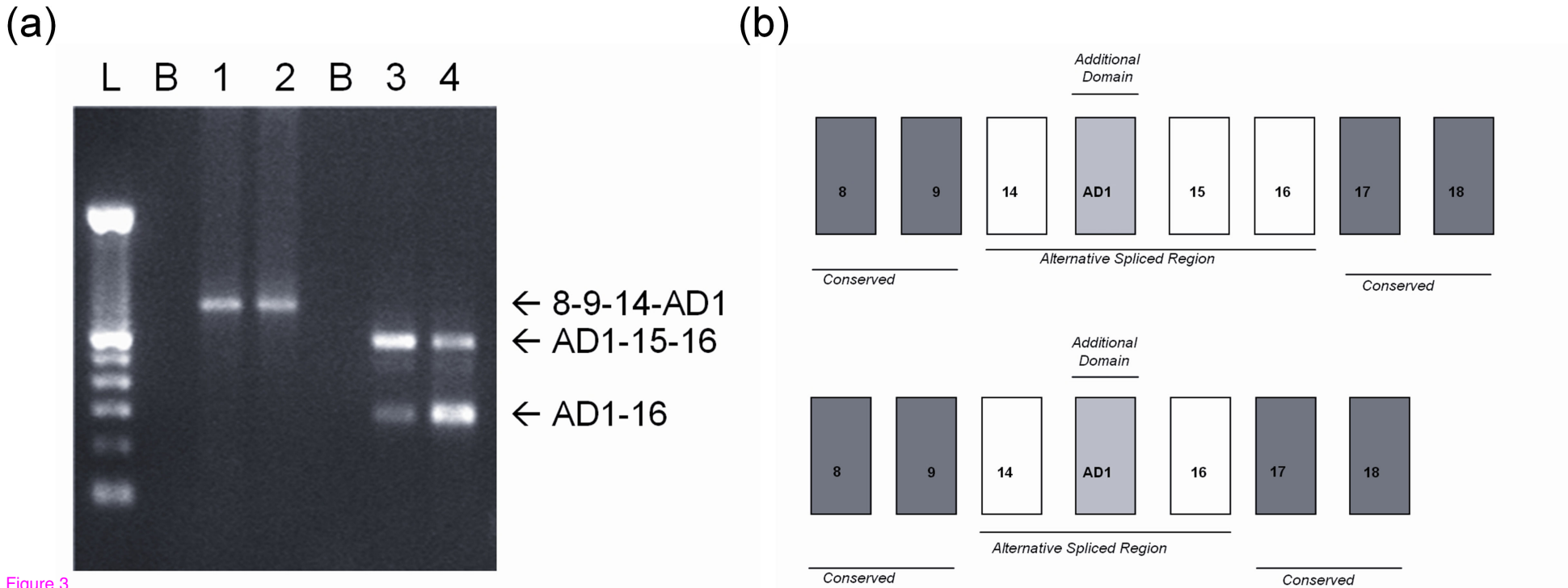
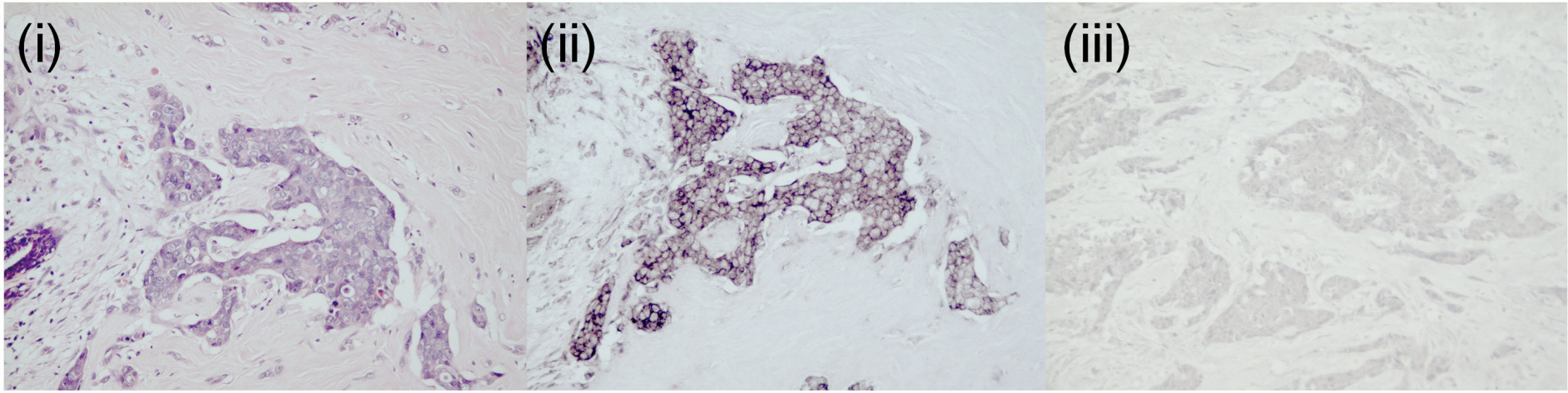

(a)
L B 1 2 B 3 4
← 8-9-14-AD1
← AD1-15-16
← AD1-16
(b)
Additional Domain
8 9 14 AD1 15 16 17 18
Alternative Spliced Region
Conserved
Conserved
Additional Domain
8 9 14 AD1 16 17 18
Alternative Spliced Region
Conserved
Conserved

Figure 3

(a)



(b)

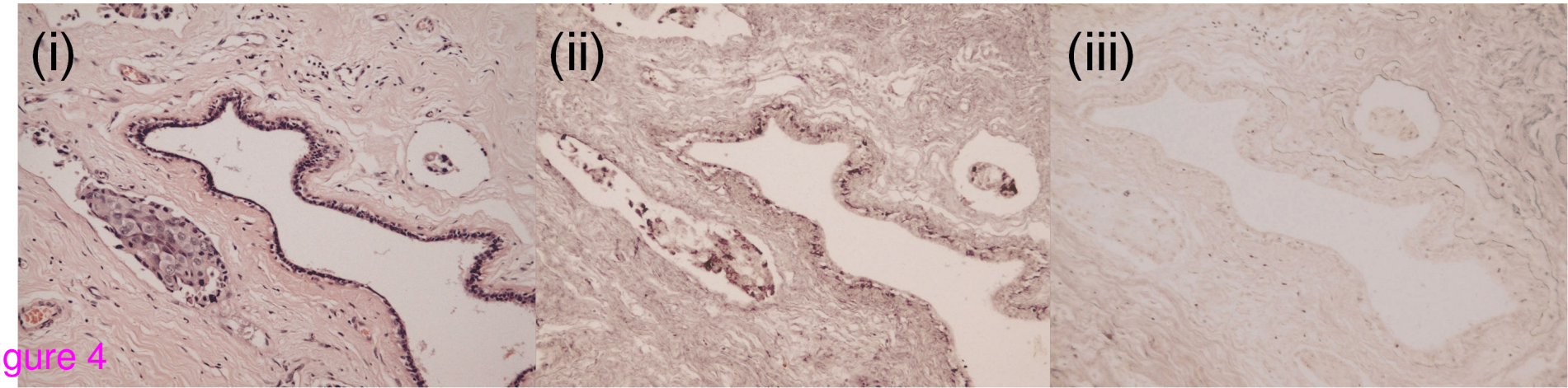


Figure 4

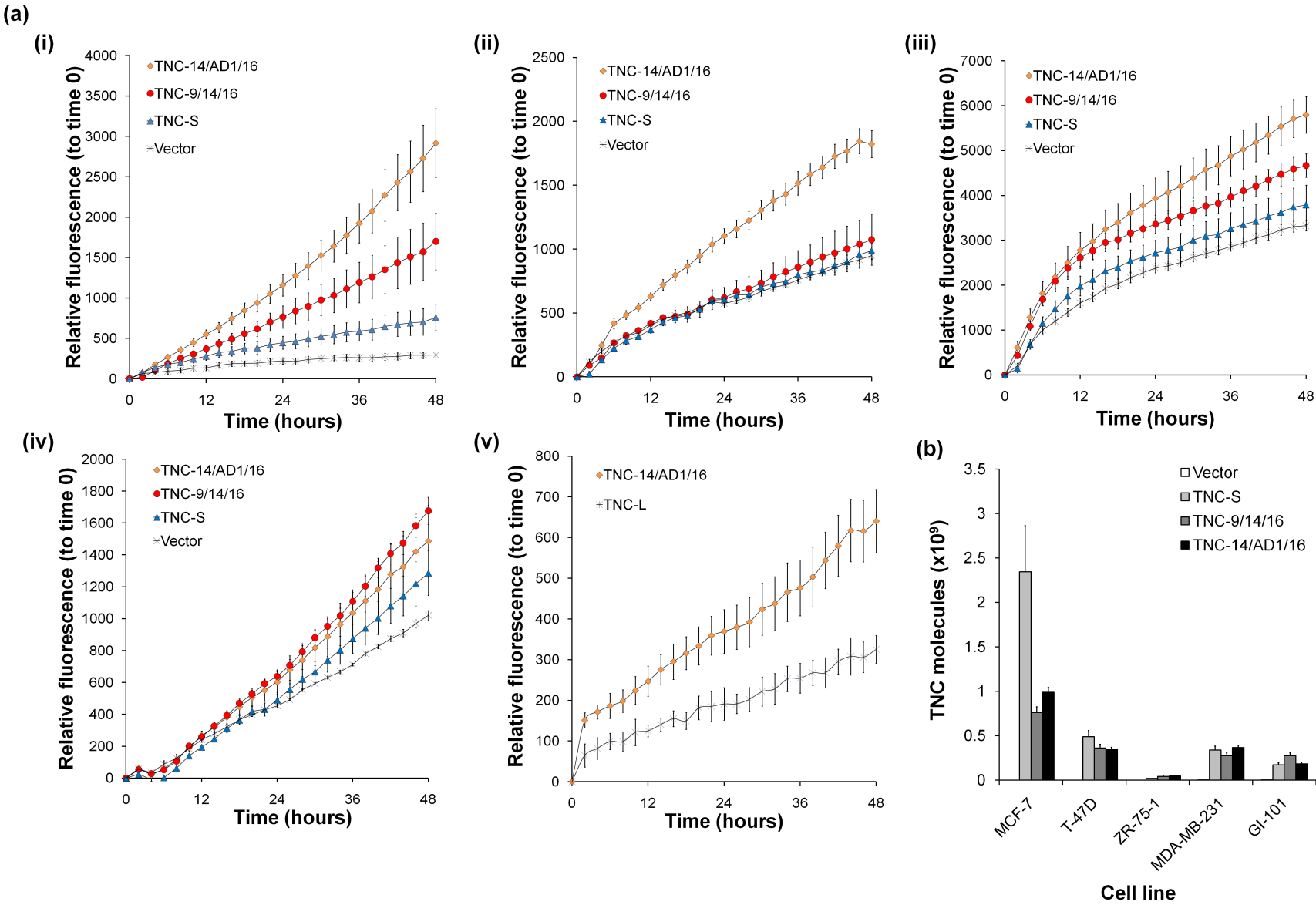

(a)
(i)
Relative fluorescence (to time 0)
Time (hours)
TNC-14/AD1/16
TNC-9/14/16
TNC-S
Vector
(ii)
Relative fluorescence (to time 0)
Time (hours)
TNC-14/AD1/16
TNC-9/14/16
TNC-S
Vector
(iii)
Relative fluorescence (to time 0)
Time (hours)
TNC-14/AD1/16
TNC-9/14/16
TNC-S
Vector
(iv)
Relative fluorescence (to time 0)
Time (hours)
TNC-14/AD1/16
TNC-9/14/16
TNC-S
Vector
(v)
Relative fluorescence (to time 0)
Time (hours)
TNC-14/AD1/16
TNC-L
(b)
TNC molecules (x10⁹)
Vector
TNC-S
TNC-9/14/16
TNC-14/AD1/16
MCF-7
T-47D
ZR-75-1
MDA-MB-231
GI-101
Cell line

Figure 5

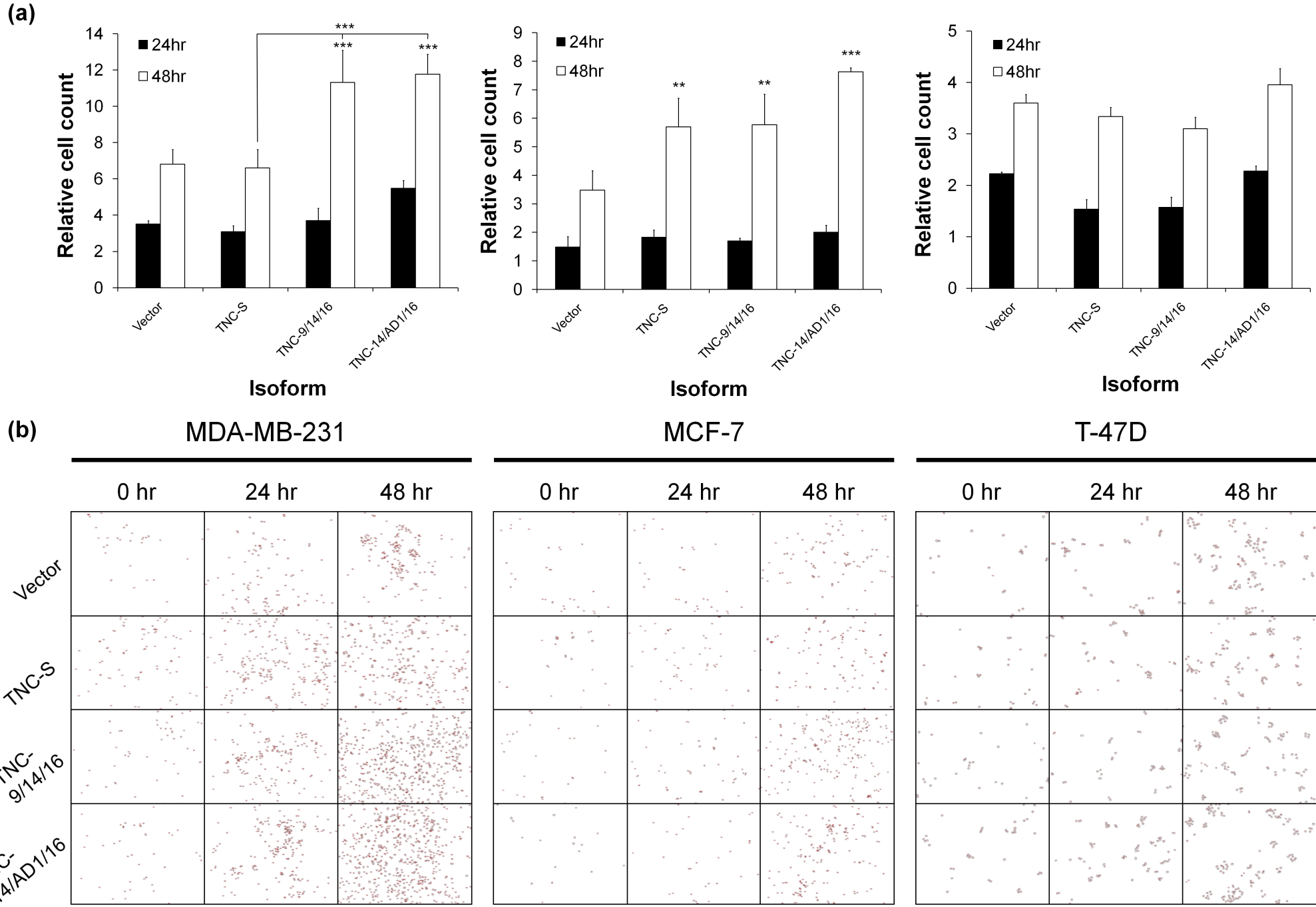

(a)
Relative cell count
24hr
48hr
***
***
***
Vector
TNC-S
TNC-9/14/16
TNC-14/AD1/16
Isoform
Relative cell count
24hr
48hr
**
**
***
Vector
TNC-S
TNC-9/14/16
TNC-14/AD1/16
Isoform
Relative cell count
24hr
48hr
Vector
TNC-S
TNC-9/14/16
TNC-14/AD1/16
Isoform
(b)
MDA-MB-231
MCF-7
T-47D
0 hr
24 hr
48 hr
Vector
TNC-S
TNC-9/14/16
TNC-14/AD1/16

Figure 6

Additional files provided with this submission:

Additional file 1: SuppFig1.pdf, 94K
http://breast-cancer-research.com/imedia/1855331654421948/supp1.pdf