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Generation and Validation of Antibodies Targeting AD1 Containing Isoform of Alternatively Spliced TNC

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Abstract Tenascin-C (TNC), a matricellular protein. TNC-AD1 isoform is molecular weight (MW) isoform has been shown to be over-expressed in high grade carcinoma hormone insensitive breast cancers in younger women. The aim of this study was to generate antibodies targeting the AD1 domain for use in tissue and functional studies. Bioinformatics analysis was preformed to select sequences within the AD1 domain for targeting with anti-peptide antibodies. Subsequently, the effect of siRNA-mediated knockdown of AD1-containing isoforms of TNC was carried out to analyse to validate the antibody specificity. In addition, the successful antibody for the detection of TNC-AD1 specify was also tested in breast cancer tissue. Immunocytochemistry and Immunohistochemistry analysis showed specific reactivity for the generated antibody with staining found to be cytoplasmic in cell lines and both the cytoplasm and ECM in breast cancer tissues; whereas western blot analysis showed no immunoreactivity detected for TNC-AD1 expression using the anti-AD1 antibodies. In conclusion, the generated antibody against TNC-AD1 was successfully recognised TNC-AD1 may serve as a specific tool for further functional studies of the pathological role of TNC-AD1 in breast cancer.

Keywords: *TNC, AD1*

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1. Introduction

Tenascin-C (TNC) is extracellular matrix (ECM) glycoprotein with high molecular weight that contains six monomers linked by disulfide bonds at their N-termini [1]. TNC transcripts containing the additional domain 1 (AD1) expressed in tumour cells of Ductal carcinoma in-situ (DCIS) and associated myoepithelial cells lining larger histologically normal breast ducts. AD1 containing transcripts were also identified in carcinomas and correlated with high grade and hormone insensitive breast cancers in younger women (< 40 years of age) [2]. However, analysis of TNC isoforms containing the AD1 domain at the protein level is lacking due to the absence of an available anti-AD1 antibody. Although a number of TNC specific antibodies are available, which target different domains (BC-24 antibody binds to Epidermal growth factor (EGF) like repeat; H-300 binds to Domain 6 and 7 of FNIII repeat and Clone IIIB binds to Domain B of FNIII repeat, none are specific to the AD1 domain. The aim of this research was to generate antibodies targeting the AD1 domain for use in tissue and functional studies.

2. Materials and Methods

Four peptides corresponding to different regions within TNC-AD1 exon were chemically synthesised. The synthesised peptides were fused to an N-terminal with a maltose binding protein (MBP) tag, conjugated to keyhole limpet haemocyanin (KLH) and immunised in rabbits. These services were carried out by Genosphere Biotechnologies, France, and Euro Genetec, Belgium. Sera was taken from pre-immune rabbits and screened for reactivity against TNC-AD1 by Western blot analysis as described in [3,4] performed using the MDA-MB-231 and HBL-100 cell lines, which both express high levels of TNC-AD1. The optimisation of the generated antibodies was also performed on cell line models and the breast carcinoma tissues using immunocytochemistry (ICC) and immunohistochemistry (IHC). Two antigen retrieval methods were carried out in order to achieve the optimal antigen retrieval for IHC staining. These methods were heat-induced epitope retrieval and enzymatic retrieval. Heat-induced antigen retrieval was performed using microwave. Briefly, slides were immersed in 1x 10mM

citrate buffer (pH 6.0) and then microwaving at 750 watt for 20 mins (Tecnolec® Superwave 750). Slides were cooled down at room temperature and then washed in ultra-pure (UP) water. Enzymatic retrieval was performed using proteinase K (PK). Briefly, sections were covered with PK at a concentration of 1μg/ml for cyto-blocks and 7μg/ml for tissue-blocks, and then incubated at 37 ℃ for 1 hr. Slides were washed and rinsed with tap water. Immuno-staining was performed via enzymatic antigen retrieval using PK followed by NovoLink™ Polymer Detection System. Briefly, peroxidase block was added into slide and incubated for 5 mins in order to inhibit the activity of endogenous peroxidase. Slides were then washed in TBS twice for 5 mins each wash. Protein block was then added and incubated for 5 mins in order to reduce non-specific binding to the primary antibody. After washing slides in TBS twice for 5 mins, samples were incubated with primary antibody diluted in 3% BSA blocking solution overnight at 4℃. After overnight incubation, slides were again washed twice in TBS for 5 mins. Post-primary block was added and incubated for 30 mins in order to enhance the penetration of 3, 3'-diaminobenzidine tetrahydrochloride (DAB) substrate buffer, and then slides were washed twice in TBS for 5 mins. NovoLink™ polymer (Anti-mouse/rabbit IgG-Poly-HRP reagent) was added to slides and incubated for 30 mins. Slides were washed in TBS twice for 5 mins. Peroxidise activity was developed with 100 μl DAB working solution (5 μl of DAB chromogen to 100 μl NovoLink™ DAB substrate buffer) and incubated for 5 mins. Slides were rinsed with tap water and stained with Mayer's Haematoxylin for 30 seconds. Sections were dehydrated with 95%, 99%, and 99% IMS for 3 mins each followed by xylene twice for 3 mins each. Finally, slides were demounted in aqueous mountant.

3. Results

3.1. AD1 Sequence Alignment

The amino acid sequences of AD1 from different species were aligned to assess their similarity and identify the correct species and protein sequence prior to antibody production. In addition, comparisons were performed between the AD1 DNA and protein sequences and other exons within the TNC FNIII-like domains in order to avoid any potential cross reactivity. Alignment of FNIII-like domains including AD1 was performed using the EMBL-EBI tools ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2). Domain C (exon 15) showed highest identity with AD1 at both the protein and DNA levels (56.18%) (Table 1).

3.2. Alignment of the Human AD1 Sequence with AD1 from other Species

The Basic Local Alignment Search Tool (BLAST) was used to analyse TNC-AD1 similarity across species including mouse, rat and chicken. BLAST analysis showed that the amino acid sequence of TNC-AD1 is conserved with 33% identity with rat AD1 (Table 2).

Table 1. Summary of TNC-AD1 sequence identity compared to other FNIII domains of TNC

Domain	Exon	Amino acid identity %	DNA identity %
A_1	10	27.17	54.13
A_2	11	26.09	51.23
A_3	12	31.52	53.90
A_4	13	30.43	50.34
B	14	33.70	55.12
C	15	35.87	56.18
D	16	29.35	52.82
AD2	AD2	29.35	52.40

Table 2. Summary of TNC-AD1 sequence identity in other species compared to the human TNC-AD1 sequence

Species	Length	Amino acid identity %	DNA identity %
Mouse	91	32	32
Rat	91	33	32
Pig	91	30	27
Chicken	91	29	34

3.3. Optimisation of TNC-AD1 Antibodies

Two polyclonal anti-peptide antibodies generated and purified by Genosphere Biotechnologies was performed. Two peptide antigens were synthesised and conjugated with KLH carrier protein (Table 3), which were then injected into two different rabbits and subjected to four cycles of immunisation. In this program, Genosphere Biotechnologies offered a complete service including total IgG purification post-immunisation. Optimisation of the purified polyclonal antibodies against TNC-AD1 was performed using both Western blot and ICC.

Table 3. Summary of the selected TNC-AD1 sequences for antibody generation

Company	Sequence	Amino acid Sequence	Amino acids length	Start position
Genosphere Biotechnologies (GB)	GB 1	CKIVINVSDAHSLHE	15	32
	GB 2	CVSVAGTTLAGDPTR	15	70

3.3. Anti-TNC-AD1 Optimisation on Cell Lines

3.3.1. Immunocytochemistry (ICC)

To assess the optimal antibody concentration, a serial dilution was performed (Table 4). The different antibody dilutions were tested on cytospun HBL-100 cells (which express high levels of TNC-AD1. Analysis of TNC-AD1 staining at a concentration of 0.5 μg/ml showed the optimal dilution and revealed clear specific cytoplasmic staining.

ICC optimisation of TNC-AD1 expression was also carried out on cells that endogenously express TNC (MDA-MB-231 and Sk-Mel-28) and a TNC null cell line

(MCF-7) was used as a negative control. ICC analysis of anti-AD1 polyclonal antibodies generated from animal-1 showed a clear detection of TNC-AD1 in cells endogenously expressing TNC-AD1 (Figure 1 A & C), with no TNC-AD1 expression detected in MCF-7 cells (Figure 1 B). Conversely, there was no detection for TNC-AD1 expression obtained from anti-AD1 polyclonal antibodies generated from animal-2 in cells endogenously expressing TNC-AD1 (Figure 1 D & F), and non-specific staining was detected in MCF-7 cells (Figure 1 E).

Table 4. Serial dilution of the purified TNC-AD1 antibody (GB1 sequence)

Antibody dilutions	Nuclear staining	Cytoplasmic staining
1:625 (4 µg/ ml)	+	+
1:1250 (3 µg/ ml)	+	+
1:2500 (2 µg/ ml)	+	+
1:5000 (1 µg/ ml	+	+
1:10,000 (0.5 µg/ ml)	-	+
1:20,000 (0.25 µg/ ml)	-	-

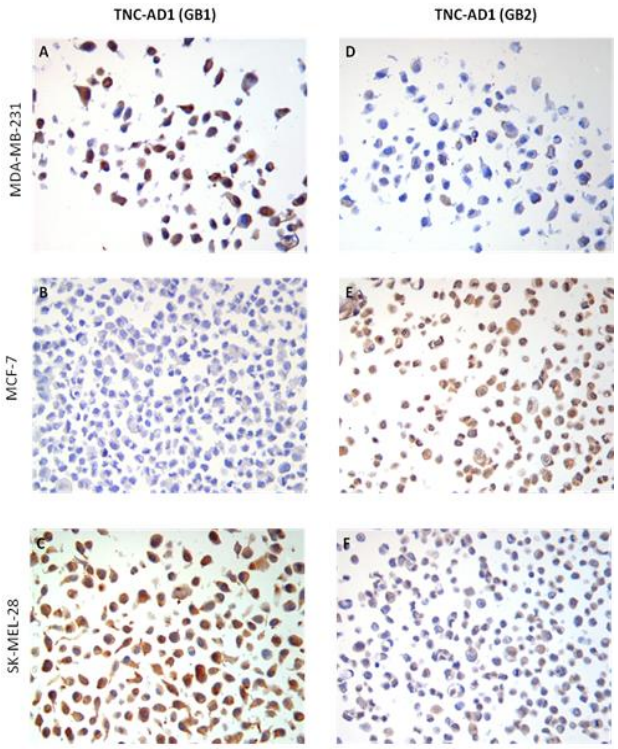

Figure 1. ICC staining for TNC-AD1 expression in cell lines. MDA-MB-231(A&D) and SK-Mel-28 (C&F) cells endogenously express TNC-AD1, whereas MCF-7 cells are TNC null (B&E). The purified antibodies generated from two immunised animals (GB1 (A,B and C); GB2 (D,E and F)) were tested for the expression of TNC-AD1. GB1 showed a clear detection of TNC-AD1, whereas GB2 did not show any expression of TNC-AD1.

The above optimisations confirmed that one of the two raised anti-AD1 polyclonal antibodies was suitable due to the detection of a signal, which was more likely against TNC-AD1. Therefore, the successful anti-AD1 polyclonal antibody was subjected to further optimisation in knockdown experiments and tissue studies.

3.3.2. Knockdown of TNC Isoforms Containing AD1

In order to test the specificity and efficiency of the purified anti-AD1 antibody to detect any loss of endogenously expressed AD1 containing TNC isoforms, MDA-MB-231 cells were transfected with specific siRNAs to total TNC and high MW isoforms containing AD1. The expression of total TNC and AD1 containing isoforms was analysed by ICC using the BC-24 anti-TNC and TNC-AD1 antibodies. Total TNC staining was less intense when stained by both antibodies in cells transfected with total TNC siRNA compared to cells transfected with a scrambled siRNA negative control (Figure 2 B & E). Interestingly, staining intensity using the anti-AD1 antibody was reduced in cells transfected with the TNC-AD1 siRNA compared to cells transfected with the scrambled sequence (Figure 2 F); whereas total TNC expression appeared to remain unchanged when stained with the BC-24 antibody (Figure 2 C). These findings suggest that the purified TNC-AD1 antibodies were able to specifically detect the endogenous AD1 containing isoforms.

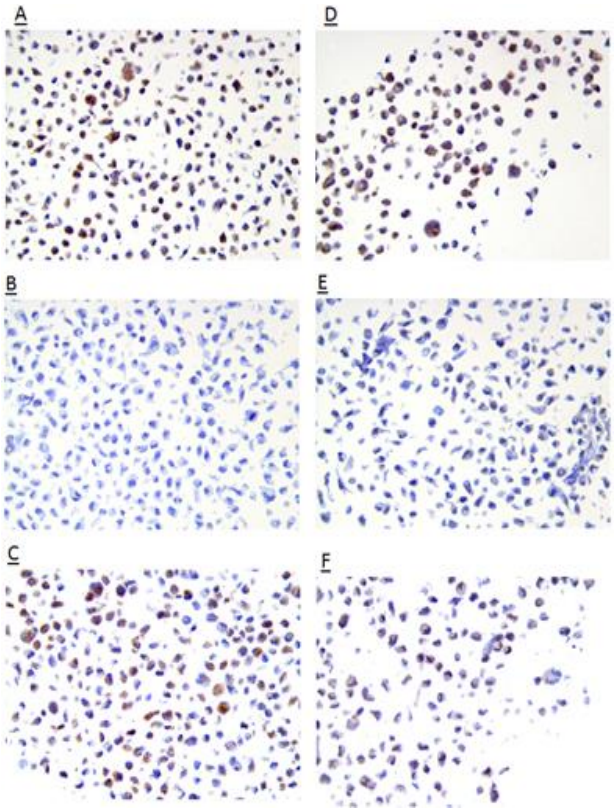

Figure 2. The knockdown of total TNC and TNC-AD1 in MDA-MB-231 using siRNA. A + D) MDA-MB-231 transfected with scrambled siRNA. B+ E) MDA-MB-231 transfected with total TNC siRNA. C+ F) MDA-MB-231 transfected with TNC-14-AD1 siRNA IHC staining using BC-24 monoclonal anti-TNC antibody is shown in A, B and C; whereas IHC staining using the polyclonal anti-TNC-AD1 antibody is shown in D, E and F.

3.3.3. Western Blot

Western blot analysis was performed using the polyclonal anti-AD1 antibodies on conditioned media from MDA-MB-231 cells and TNC null MCF-7 cells transiently transfected with recombinant TNC isoforms TNC-Short, TNC-Long, TNC-9/14/16, TNC-14/AD1/16 and an empty vector. Western blot analysis of MCF-7 conditioned

media using the H-300 anti-TNC antibody confirmed successful transfection (Figure 3A), as well as expression of TNC protein in MDA-MB-231 cells and an absence in non-transfected MCF-7 cells (Figure 3B); however, no immunoreactivity was detected for TNC-AD1 expression using the anti-AD1 antibodies by Western blot, in MDA-MB-231s or MCF-7 cells transiently transfected with TNC-14/AD1/16.

3.4. Anti-TNC-AD1 Optimisation on Tissues

Following successful immunoreactivity of the purified anti-AD1 polyclonal antibody using cell lines, subsequent analysis of the anti-AD1 polyclonal antibody (GB1) was performed on breast tissue by IHC. Optimal conditions for IHC using the anti-AD1 antibody were determined by testing different antigen retrieval methods.

3.4.1. Antigen Retrieval

Two antigen retrieval methods were tested to allow efficient binding of the antigenic sites. These methods were heat-induced epitope retrieval using a microwave and enzymatic retrieval using proteinase K. FFPE tissue from DCIS samples positive for TNC-AD1 mRNA by RT-qPCR were analysed by IHC with the anti-AD1 polyclonal antibody, and BC-24 anti-TNC monoclonal antibody was used as a positive control for total TNC staining. Antigen retrieval using proteinase K showed clear expression of TNC-AD1 in the tumour stroma as well as in the myoepithelial layer of the breast duct and blood vessels (Figure 4 B & D). However, considerable non-specific staining was observed in the tumour cells using anti-AD1 antibody when compared to BC-24 anti-TNC. In contrast, heat-induced epitope retrieval using a microwave only revealed weak staining intensity (Figure 4 A & C).

3.4.2. Expression of AD1 in Breast Tissue

Having validated the specificity of the anti-AD1 antibody by ICC in cell lines, the TNC-AD1 antibody was used to analyse AD1 protein expression in normal breast and breast carcinoma tissues. Analysis of TNC expression using the BC-24 TNC antibody showed TNC to be localised to myoepithelial cells in normal breast tissue, which is likely to be the fully truncated TNC isoform (Figure 5A); whereas AD1 was not detected in any normal breast tissue analysed using the anti-AD1 antibody (Figure 5 B). Figure 5 C, D and E are examples of breast cancer tissues which were screened for mRNA expression of AD1 containing TNC isoforms using RT-qPCR and were also found to be consistent with AD1 protein expression in this study using IHC and the anti-AD1 antibody.

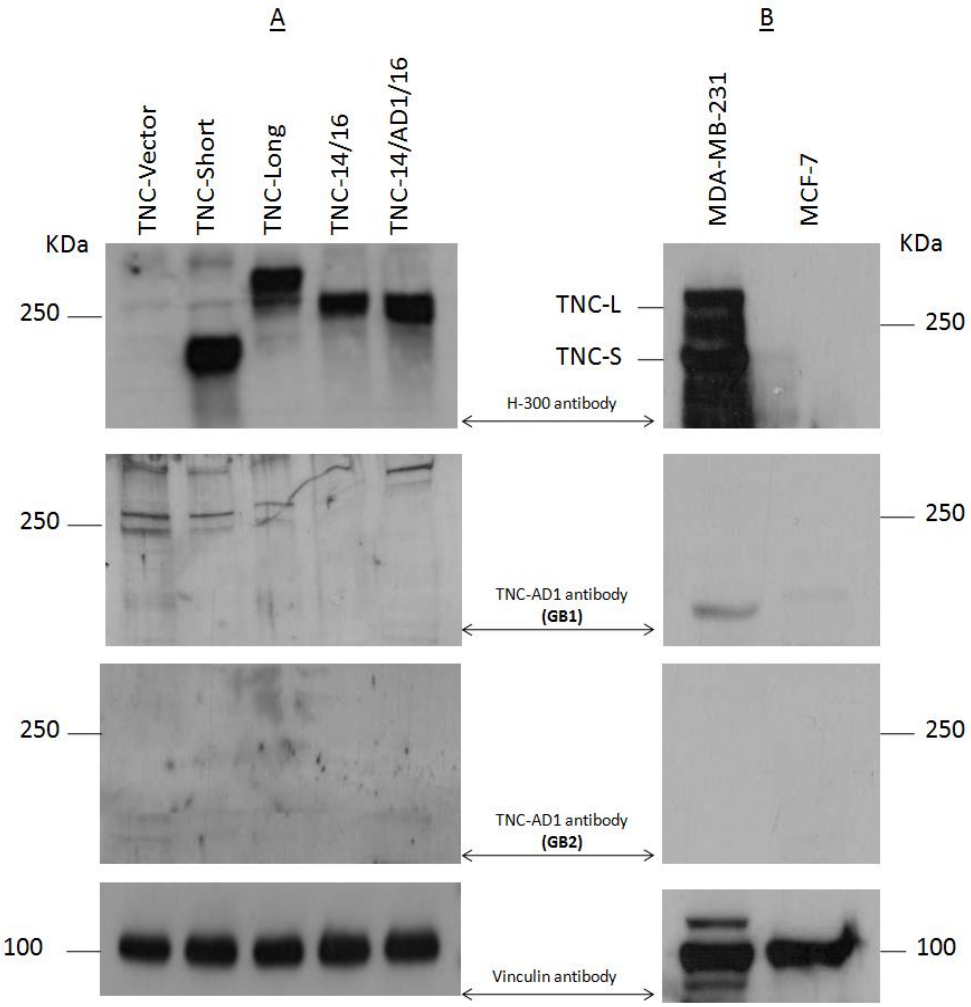

Figure 3. Western blot analysis of TNC expression using H-300 anti-TNC and two anti-AD1 antibodies. A) Expression of exogenous TNC isoforms transfected in MCF-7. B) Expression of endogenousTNC using MDA-MB-231 cell line compared to MCF-7 TNC null cell lines. Anti-vinculin antibody was used as loading control.

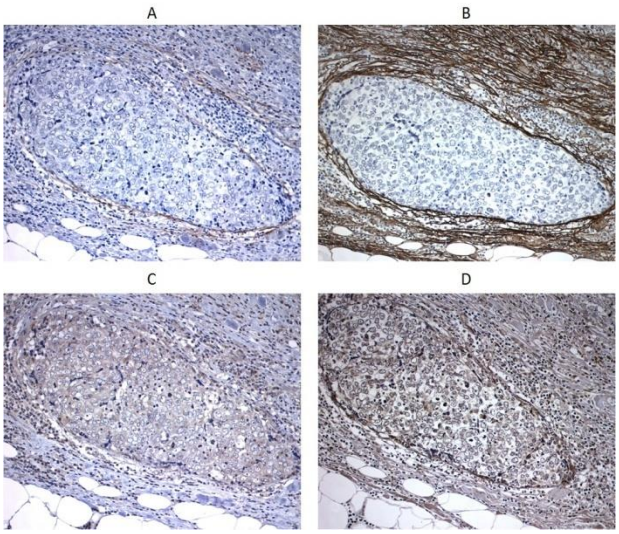

Figure 4. IHC optimisation of antigen retrieval methods. Heat-induced epitope retrieval is shown in (A and C), enzymatic retrieval is shown in (B and D). TNC-AD1 antibody staining is shown in (C and D), BC-24 anti TNC antibody staining is shown in (A and B).

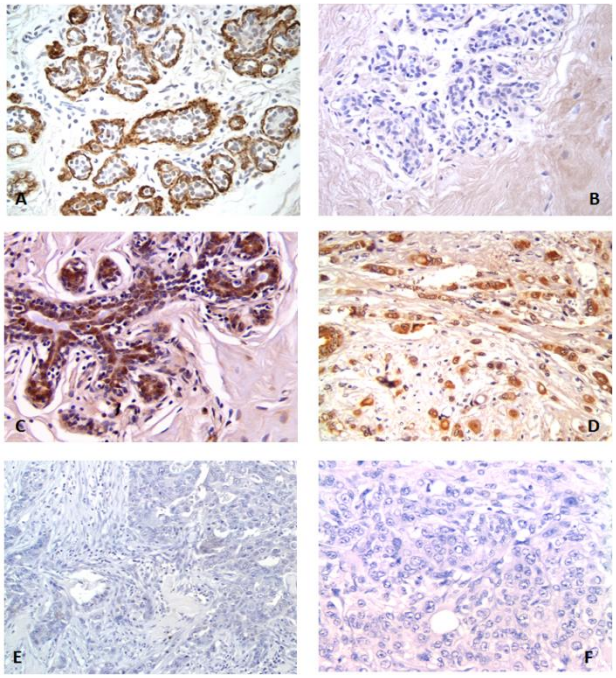

Figure 5. IHC staining of breast tissues using BC-24 anti-TNC antibody (A), and anti-AD1 antibody (B; C; D and E). A) Truncated TNC expression in normal breast. B) TNC-AD1 negative in normal breast tissue. C) TNC-AD1 positive in fibroadenoma breast tissue breast tissue. D) TNC-AD1 positive in lobular carcinoma breast tissue. E) TNC-AD1 negative breast tissue. F) No primary antibody control.

4. Discussion

Of the generated polyclonal antibodies, only one successfully recognised TNC-AD1 when tested by ICC and IHC, but not by Western blot. The antibody may not work in a particular assay, which may be that the peptide sequence corresponds to a non-exposed region of the native protein [5] or the epitope for antibody recognition was destroyed during the denaturation stage of the Western blot protocol. Furthermore, commercially available anti-TNC antibodies are also known to only work when tested by Western blot or ICC/IHC (e.g. H-300 and BC-24, respectively). In this study, the expression of TNC-AD1 was found to be cytoplasmic in cell lines endogenously expressing TNC-AD1, and both the cytoplasm and ECM in breast cancer tissues. The IHC method is commonly used in diagnostic applications despite its weaknesses including the sensitive variations between fields, fixation time and processing, antigen retrieval, staining procedure and interpretations [6]. On the other hand, the sensitivity of quantitative RT-PCR is capable of detecting low abundance mRNA [7]. Therefore, the correlation between the expressions of TNC-AD1 using both methods is recommended using large cohort study to minimise the variations due to sample heterogeneity resulted from the use of fresh frozen (FF) and FFPE tissues. Hence, detecting specific TNC-AD1 isoforms using antibody targeting was achieved, and in future this novel polyclonal antibody could be used in addition to commercially available antibodies (ie BC24, H300 and clone IIIB) for TNC detection in normal and malignant tissues.

5. Conclusion

The generated polyclonal antibody against TNC-AD1 successfully recognised TNC-AD1 and was used in a pilot study to evaluate expression of TNC-AD1 protein in cell lines human breast tissues. The AD1 antibody may serve as a specific tool for further functional studies of the pathological role of TNC-AD1 in breast cancer.

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