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Investigation of Tenascin-C Effects on Thrombospondin 1 in Highly Invasive Breast Cancer Cell Lines

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Abstract Breast cancer is the most diagnosed cancer among women worldwide. Tenascin-C (TNC) is a high glycoprotein which has been shown to be over-expressed in the breast cancer stroma and promotes cancer progression. Thrombospondin 1 (TSP-1) is an adhesive glycoprotein which has been shown to play roles in platelet aggregation, angiogenesis, and tumorigenesis. The aim of this study was to investigate the effects of TNC knockdown in TSP-1 expression in highly invasive breast cancer cell lines. Small interfering RNA (siRNA) targeting total TNC and high molecular TNC isoforms (TNC-14 and TNC-14-AD1) were transfected into the highly invasive MDA-MB-231 breast cancer cell line. The alterations caused by TNC knockdown on TSP-1 were analysed at protein level by Western blot using conditioned media. The expression of TSP-1 was significantly up-regulated as a result of TNC down-regulation. In conclusion, TNC knockdown significantly increases TSP-1 expression, confirming their importance in tumorigenesis.

Keywords: *TNC, TSP-1, siRNA, knockdown*

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1. Introduction

Breast cancer is the most common cancer among women worldwide. Approximately 1.7 million women were diagnosed with breast cancer in 2012. (www.wcrf.org/). In addition, around 521,000 deaths were recorded in 2012 with variations across the regions in the world due to better survival (WHO). Breast cancer is complicated due to molecular signatures and diverse genetic alterations; each with distinct clinical outcomes [1]. Tenascin-C (TNC) is extracellular matrix glycoprotein which has shown to promote cell migration, inhibit focal adhesion formation, induce cell proliferation, and act as a cell survival factor as well as promoting angiogenesis and remodelling of ECM components [2]. TNC knockdown has shown to reduce cell invasion and proliferation [3]. TNC knockdown has also shown to regulate genes associated with tumor progression [4]. The thrombospondin 1 (TSP-1) is a homologous protein which regulates cellular phenotype and ECM during tissue remodeling that associated with wound healing and tumor progression [5]. Alteration in the oncogenic activity and tumor suppressor genes result in reduced expression of TSP-1 and the acquisition of an angiogenic phenotype [6,7]. In breast cancer, TSP-1 expression is induced by stromal fibroblasts, macrophages and endothelial cells in breast cancer [8]. TSP-1 may inhibit tumor angiogenesis by down-regulating circulating endothelial cell progenitors [9,10].

The aim of the present study was to knockdown TNC in highly invasive breast cancer cell line to determine the effect of TNC knockdown on TSP-1 expression. The results of the study showed that TNC silencing significantly increased the expression of TSP-1 which required further study to address their effects on tumor behaviors.

2. Materials and Methods

The transfection of breast cancer cell line MDA-MB-231 with synthetic siRNA targeting total TNC and TNC isoforms was performed following our previous guidelines [3], and scrambled siRNA as a negative control. Western blot was also performed to determine the amount of extracellular levels of total TNC and TNC isoforms. 24 hours post transfection; completed media was replaced with serum-free media and cells were incubated for 48 hours. Cell conditioned media (CM) was collected for analysis of secreted protein after incubation with Opti-MEM media for 48 hrs and centrifuged at 1000 rpm for 5 mins. 2 ml of CM was placed into the top of a Centricon column and then centrifuged at 4000 rpm in a bench top centrifuge

(Jouan) for 30 mins at 4°C. The column was inverted into a clean collection tube and stored at -20°C until required. The concentration of the collected CM was measured using the bovine serum albumin protein assay. Protein was run on 6% SDS-PAGE gels and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). Membrane was blocked for one hour using blocking solution containing Tris-buffered saline, 5% milk and 1% Tween. Primary antibody (clone H300, rabbit polyclonal TNC antibody; Santa-Cruz Biotechnology, Santa Cruz, CA, USA); (TSP-1 (A6.1) monoclonal antibody for TSP-1; Santa-Cruz Biotechnology, Santa Cruz, CA, USA) were added and incubated for overnight at 4°C. After subsequent washes, secondary antibody (donkey anti-rabbit horseradish peroxidase-linked IgG; Amersham Biosciences) was added and incubated for 1 hour. An ECL kit was used for detection of protein according to manufacturer's instructions and visualised using X-ray film (Xerox, USA) with a variety of exposure times.

3. Results

3.1. Transfection of MDA-MB-231 Cell Lines

The effect of TNC knockdown on TSP-1 expression was investigated in MDA-MB-231 cell lines. Three replicate transfections were performed using the siRNA targeting exon 24 of TNC and scrambled siRNA. The transfection efficiency of total TNC knockdown was confirmed by Western blot using the H-300 anti-TNC antibody. Western blot analysis of CM collected from cells transfected with siRNAs targeting total TNC confirmed down regulation of TNC at the protein level compared to cells transfected with scrambled siRNAs (Figure 1), as shown by the absence of the two predominant isoforms (TNC-L and TNC-S).

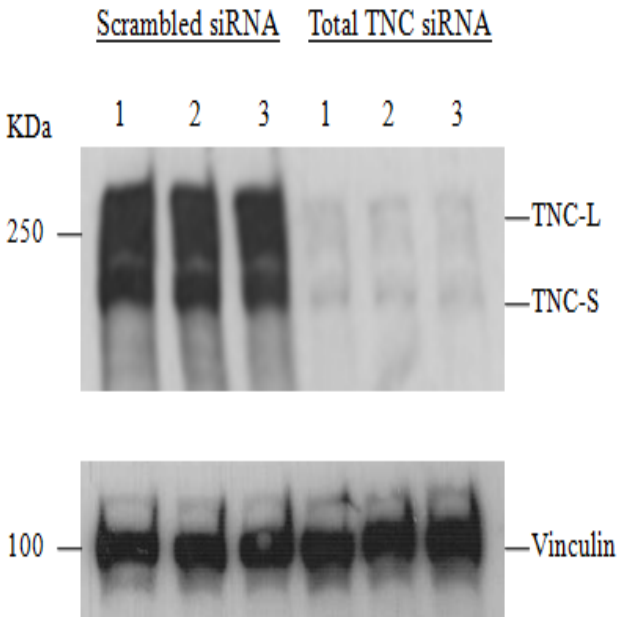

Figure 1. Western blot analysis of conditioned media from MDA-MB-231 breast cancer cell lines transfected with siRNA targeting total TNC and a scrambled siRNA. The upper panel shows loss of expression of the predominant TNC isoforms (TNC-L and TNC-S). The lower panel shows the loading control (Vinculin).

3.2. TSP-1 regulation by Western Blot

Thrombospondin 1 (TSP-1) was selected as it was most significantly down-regulated in T98G glioblastoma cells cultured on a TNC milieu compared to cells cultured on fibronectin using microarray studies [11]. Western blot analysis of TSP-1 expression was carried out using the same cell conditioned media collected from three independent biological transfections of MDA-MB-231 cells with both total TNC and scrambled siRNAs. Western blot analysis showed increased expression of TSP-1 after TNC knockdown (Figure 2).

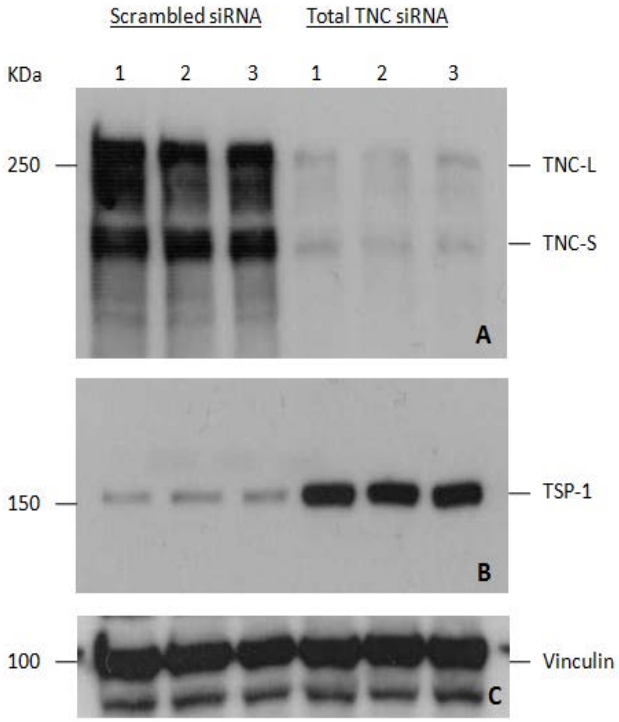

Figure 2. Western blot analysis in conditioned media of MDA-MB-231 breast cancer cell lines transfected with siRNA targeting total TNC and scrambled siRNA. A) H300 anti-TNC antibody reactivity confirmed the down-regulated expression of predominant TNC isoforms (TNC-L and TNC-S). B) Anti-TSP-1 reactivity showed the up-regulated expression of TSP-1 in cells transfected with total TNC siRNA. C) Loading control (Vinculin).

3.3. Effects of TNC Isoform Knockdown on TSP-1 Expression

To analyse the effect of specific TNC isoform knockdown on TSP-1 expression, MDA-MB-231 cells were transfected with siRNAs targeting high MW TNC isoforms (TNC-14 and TNC-14-AD1) and incubated for 72 hours. Western blot was performed to test the efficiency of TNC high MW isoform knockdown, and assess whether there are different effects on TSP-1 expression as a result of specific TNC isoform knockdown. Western blot analysis of conditioned media collected from cells transfected with siRNAs targeting total TNC and TNC high MW isoforms confirmed TNC down-regulation at the protein level using H-300 anti-TNC antibody.

However, there was a specific reduction of the high MW TNC level caused by siRNA targeting exon 14 with no effect on truncated TNC levels. There was no observable change in TNC protein levels in cells transfected with siRNAs against exon 14-AD1 (Figure 3 A) as might be expected, TNC-L and TNC-S were the predominant bands detected. In addition, Western blot analysis of TSP-1 expression in cells transfected with total TNC and high MW isoforms confirmed TSP-1 up-regulation in cells transfected with siRNAs targeting total TNC and TNC high MW isoforms compared to cells transfected with scrambled siRNA (Figure 3B).

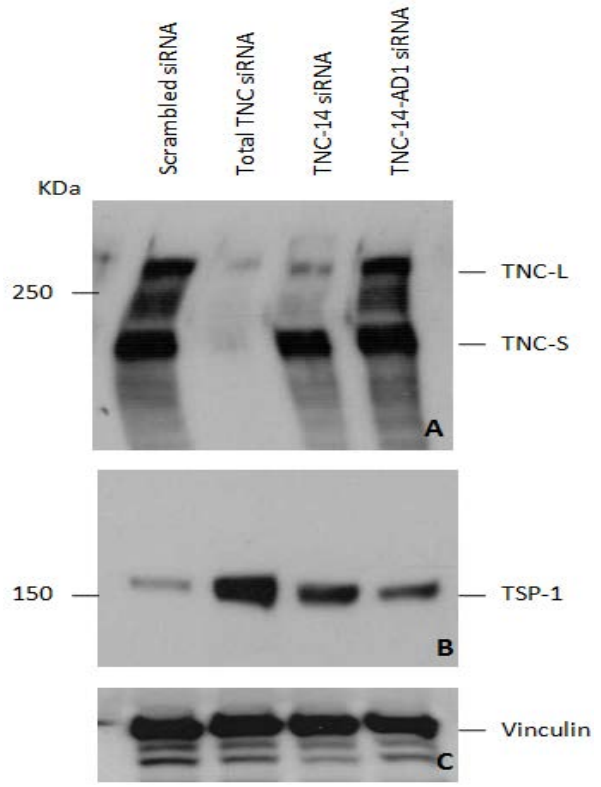

Figure 3. Western blot analysis in conditioned media of MDA-MB-231 breast cancer cell lines transfected with siRNA targeting high MW TNC isoforms, total TNC and scrambled siRNA. A) H300 anti-TNC antibody reactivity confirmed the down-regulated expression of total TNC and high MW TNC isoforms. B) Anti- TSP-1 reactivity showed the up-regulated expression of TSP-1 in cells transfected with total TNC and high MW TNC isoforms siRNAs. C) Loading control (Vinculin)

4. Discussion

TSP-1 has pro-angiogenic activity in breast cancer and circulating plasma levels of TSP-1 has been suggested as a marker of breast cancer aggressiveness [12]. Although TSP-1 was found to be low in tumour cells, fibroblasts in the stroma secrete higher levels of TSP-1 and inhibit angiogenesis [13,14]. Stromal TSP-1 expression was positively correlated to the ECM expression of tenascin, laminin, fibronectin, syndecan-1 and collagen type IV [15]. Ioachim et al (2012) examined tissue sections of 124 breast carcinomas for TSP-1 expression and compared this to clinical parameters, angiogenesis and ECM protein expression (i.e. tenascin). In the survival analysis, lower TSP-1 tumour expression was associated with increased risk of recurrence, whereas higher TSP-1 expression was found in invasive lobular breast cancer [15]. In this study, the expression of TSP-1 was up-regulated as a result of TNC knockdown, suggesting that TSP-1 up-regulation may reduce the risk of recurrence. TSP-1 up-regulates the integrin alpha-6 subunit in human breast cancer cells, thereby augmenting cell adhesion to laminin and assists tumour cell invasion [16]. The transition from resting endothelial cells to a sprouting phenotype was promoted by tenascin, whereas TSP-1 inhibits sprout formation [17]. TSP-1 was also synthesised and secreted by tumour cell lines such as melanoma, squamous carcinoma, osteosarcoma and glioma [18]. The relationship between TSP-1 and TNC is unclear. In this study, there was clear evidence of the effects of TNC-AD1 silencing on TSP-1 up-regulation, which was confirmed by staining of TSP-1 on breast cancer cell lines. However, further validation is required using breast tissues to confirm this findings.

5. Conclusion

This study has confirmed that TNC knockdown by siRNA affects expression TSP-1 at protein level in conditioned media. These findings could provide a new mechanism of TNC action in tumorigenesis.

Abbreviations

Tenascin-C (TNC), Extracellular Matrix (ECM), Small Interfering RNA (siRNA), Additional Domain 1 (AD1), TNC-Long (TNC-L) ,TNC-Short (TNC-S). Molecular Weight (MW).

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