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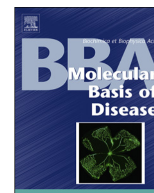

Impaired mammary tumor formation and metastasis by the point mutation of a Smad3 linker phosphorylation site

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A B S T R A C T

Triple-negative breast cancer (TNBC) is often aggressive and metastatic. Transforming growth factor-β acts as a tumor-promoter in TNBC. Smad3, a major downstream effector protein in the TGF-β signaling pathway, is regulated by phosphorylation at several sites. The functional significance of the phosphorylation of the linker region in Smad3 is poorly understood for TNBC. Among the four sites in the Smad3 linker region, threonine-179 (T179) appears to be unique as it serves as the binding site for multiple WW-domain-containing proteins upon phosphorylation, suggesting that this phosphorylation is a key for Smad3 to engage other pathways.

Using genome editing, we introduced for the first time a knock-in (KI) mutation in the endogenous Smad3 gene in IV2, a lung-tropic subline of the human MDA-MB-231 TNBC cell line. In the resulting cell line, the Smad3 T179 phosphorylation site is replaced by non-phosphorylatable valine (T179V) with the mutation in both alleles.

The T179V KI reduced cell growth rate and mammosphere formation. These phenomena were accompanied by a significant upregulation of $p21^{Cip1}$ and downregulation of c-Myc. The T179V KI also reduced cell migration and invasion in vitro. In the mouse xenograft models, the T179V KI markedly reduced the establishment of primary tumor in the mammary fat pad and the lung metastasis.

Our results using gene editing indicate the cancer-promoting role of Smad3 T179 phosphorylation in the human TNBC cells. Our findings highly suggest that controlling this phosphorylation may have therapeutic potential for TNBC.

1. Introduction

Triple-negative breast cancer (TNBC) does not contain receptors for estrogen, progesterone or HER2 [1–3]. TNBC accounts for ~15% of breast cancer cases but is often more aggressive, highly metastatic and difficult to treat [1–3]. Accordingly, most of the patients with TNBC have a poor prognosis [1–3].

Transforming growth factor-β (TGF-β) is the founding member of an evolutionally conserved large family of cytokines that control various physiological activities including cell proliferation, differentiation, migration, apoptosis, immune regulation and homeostasis in adult tissues [4–6]. TGF-β induces a cytostatic response in normal cells but in cancer cells the growth-inhibitory response to TGF-β is often malfunctioning [7–11]. Furthermore, TGF-β can also act as a prometastatic factor in cancer cells [7–12]. Smad3 is a major intracellular effector protein of the TGF-β signal and plays important roles in both of these conflicting outcomes. It has been shown that Smad3 can change its property from a tumor suppressor to a tumor promoter during cancer progression [13]. The underlying molecular basis is yet to be further elucidated.

Smad3 is regulated by phosphorylation at several sites. Its carboxyl-terminal serine residues are directly phosphorylated by the transmembrane Ser/Thr kinase receptor. The C-terminally phosphorylated Smad3 forms a heteromeric complex with Smad4, accumulates in the nucleus, where it together with other cofactors, regulates the various target genes. Availability of those cofactors and timing of the

Abbreviations: IVIS, in vivo imaging system; qPCR, quantitative polymerase chain reaction; TALEN, transcription activator-like effector nuclease; TNBC, triple negative breast cancer; FACS, fluorescence-activated cell sorting; ANOVA, analysis of variation

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interaction with them provides complexity to the Smad3 functions in various physiological contexts. Smad proteins consist of two globular domains connected by a proline-rich linker region. While the two globular domains are conserved among Smads, the linker region is more divergent in amino acid sequence. In the Smad3 linker region there are four phosphorylation sites that are followed by a proline residue (T179, S204, S208 and S213). These sites can be phosphorylated by multiple protein kinases such as MAP kinase (MAPK) superfamily members, cyclin-dependent kinases (CDKs), GSK3β and AKT [14–31]. Phosphorylation by different protein kinases under different conditions can add another layer of complexity to the Smad3 functions.

The functional significance of the Smad3 linker phosphorylation in TNBC is poorly understood. So far, several studies used overexpression of Smad3 linker phosphorylation site mutants in several types of cancer cells without reaching a consensus conclusion [32–38]. Recent development of gene editing techniques allowed us to generate cell lines with endogenously targeted gene knock-in (KI) by a mutant sequence [39–41]. With this physiologically more relevant system, we evaluated the effect of the abolishment of one of the Smad3 linker phosphorylation sites, T179. Among the four linker phosphorylation sites T179 is unique as phosphorylation of T179 seems under distinct regulation from the other three Smad3 linker sites. The phosphatases that dephosphorylate pS204, pS208 and pS213 cannot catalyze pT179 dephosphorylation [42,43]. The phosphatase that dephosphorylates pT179 has not been identified. Furthermore, Smad3 T179, but not the other three linker phosphorylation sites, can serve as the binding site for Smad3-interacting proteins such as Pin1, a proline isomerase, E3 ubiquitin ligases Smurf2 and NEDDL4, and PCBP1 that is involved in mRNA processing [27,30,35,36,44–46]. Interestingly, the putative phosphorylation site corresponding to the mammalian Smad3 T179, but not the other three sites, is also present even in a Smad ortholog of worm [47], suggesting the importance of this site.

We previously reported that the Smad3 linker phosphorylation is inhibitory to the antiproliferative function of Smad3 in normal cells [21]. In the present study we used an aggressive and lung-tropic derivative of the human MDA-MB-231 cell line, designated as IV2 [48]. The level of pT179 is significantly elevated in this in vivo-selected cell line compared to its parental line. We found that mutation of the Smad3 linker region phosphorylation site T179 to valine in the IV2 cells empowered Smad3 antiproliferative function. Furthermore, we show that the T179V cells markedly reduced tumor incidence and lung metastasis in xenograft models.

2. Materials and methods

2.1. The MDA-MB-231 IV2 subline

In vivo selected lung-tropic IV2 cell line is a human MDA-MB-231 derivative generated by Chan et al. [48] and was kindly given to us as a gift. In brief, 1×10^6 parental MDA-MB-231 cells were injected via tail vein into SCID mice and metastatic cells were isolated from lung 30 days post-injection. The isolated metastatic cells were then similarly injected and the lung metastatic cells were again recovered and amplified. The cells were cultured in DMEM with 10% FBS at 37 °C in 5% CO_2. The MDA-MB-231 cells were obtained from ATCC. Both parental and the lung-tropic subline (i.e., IV2) of MDA-MB-231 were routinely checked by DNA STR for verification. The parental IV2 cells were used as the wild type control in this study.

2.2. Targeted endogenous gene knock-in by gene editing

pZG24T03-GFP and -mCherry transcription activator-like effector nuclease (TALEN) constructs targeting left arm and right arm sequences (17 bp) surrounding the nucleotide sequence for Smad3 T179 were constructed by ZGene Biotech Inc., Taiwan. The target sequences are: left arm, TCCCAGAGACCCCACCC; right arm, CACTGGTTTCTCCATCT. The single strand oligo DNA (ssODN) sequence for mutation is: CCAAGCTGTGAAGGCCTTTTAACAGACCACCTTCCTTCTGATTCCCAGAGgtaCCtCCCCCTGGCTACCTGAGTGAAGATGGAGAAACCAGTGACCACCAGATGAACCACAGCATGGACGCAGGTCAGTCATG. Here the mutated nucleotides are shown by small cases. The mutation created a *Kpn*I restriction site (underlined) that was used as an indicator for successful knock-in. A silent mutation was introduced after the KpnI site for the sake of increasing the knock-in efficiency. No putative off-target sequences were found for either construct in the analysis using the software TAL Effector Nucleotide Targeter 2.0 (Cornell University).

The IV2 cells in 100 mm plate were transfected with 5 μg of each TALEN construct (left and right arm) and 65 pmoles ssODN by electroporation using NeonTM Transfection System Model MPK5000. The GFP-positive cells were sorted by 488nm laser in FACSAria cell sorter (BD Biosciences) and seeded on 100 mm plates at density of 1000 cells/plate. Cells from well-separated colonies were propagated. Genomic DNA was prepared and the DNA fragment around Exon 4, where the nucleotides for T179 are located, was amplified by PCR using primers: GGACAGGGGCGAGGACAAC (forward) and CAATCTCCCCCATCATCTGT (reverse). The *Kpn*I-positive PCR fragments were subjected to DNA sequencing for the Smad3 T179V targeted gene knock-in.

2.3. Cell growth and mammosphere formation assays

Cells (1×10^5) were seeded in 100 mm plate in the medium with or without 0.5 nM TGF-β (PeproTech Asia, Rehovot, Israel). Cell numbers were counted daily for 5 days. For mammosphere formation assay, 1×10^3 cells were seeded on a 96-well Ultra-low attachment surface polystyrene plate (Corning) with PSG growth medium (Thermo Fisher Scientific) supplemented with B27 (Invitrogen), 20 ng/ml EGF and 20 ng/ml bFGF (BD Biosciences), and 4 μg/ml heparin (Sigma). Mammospheres were counted after 10 days.

For cell cycle profiling, cells were untreated or treated with 0.5 nM TGF-β for 24 h. The harvested cells were subjected to fluorescence-activated cell sorting (FACS) analysis as described previously [21] using BD FACSCalibur (Argon-Ion Laser 488 nm). Flow Jo 7.6.5 software was used for the cell cycle profiling.

2.4. Gene expression analysis

For quantitative RT-PCR (qPCR), cells were treated or untreated with TGF-β for various time periods before being harvested. Total RNA was prepared by using Trizol (Invitrogen). The first strand cDNA was synthesized from 1 μg total RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). qPCR was run on CFX96 Real-Time System (BioRad) with KAPA SYBR FAST qPCR kit (KAPA Biosystems). The β-actin gene was used as an internal standard for normalization.

Luciferase assay using a TGF-β-responsive promoter construct was described previously [15,25].

2.5. Western blot

Cells were treated or untreated with 0.5 nM TGF-β for the time period indicated in the Figure Legends. The cells were harvested in PIPA buffer supplemented with 1 mM DTT, 25 ng/μl RNAse, 1 × cOmplete protease inhibitor cocktail (Roche). When phosphoprotein was analyzed, 1 × PhosStop cocktail (Roche) was also included. The cell lysate was cleared by centrifugation at 12,000 rpm for 30 min. The cleared lysate was subjected to SDS PAGE followed by Western blotting using antibodies indicated in the Figures. The Smad3 phospho-T179 antibody was described previously [15,21,25]. The sources of the other commercially available antibodies are as follows: Smad3 (clone EP568Y), Millipore; pSmad3 (C-terminus), RD Systems; p21 (clone B6B), BD Pharmingen; c-Myc (clone C1826), Clontech; β-actin (clone C4), Santa Cruz; Pin1 (*H*-123), Santa Cruz; Smurf2 (clone EP629Y3),

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