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Novel WWP2 ubiquitin ligase isoforms as potential prognostic markers and molecular targets in cancer

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ABSTRACT

The WWP2 E3 ubiquitin ligase has previously been shown to regulate TGFβ/Smad signalling activity linked to epithelial–mesenchymal transition (EMT). Whilst inhibitory I-Smad7 was found to be the preferred substrate for full-length WWP2-FL and a WWP-C isoform, WWP2-FL also formed a stable complex with an N-terminal WWP2 isoform (WWP2-N) in the absence of TGFβ, and rapidly stimulated activating Smad2/3 turnover. Here, using stable knockdown experiments we show that specific depletion of individual WWP2 isoforms impacts differentially on Smad protein levels, and in WWP2-N knockdown cells we unexpectedly find spontaneous expression of the EMT marker vimentin. Re-introduction of WWP2-N into WWP2-N knockout cells also repressed TGFβ-induced vimentin expression. In support of the unique role for WWP2-N in regulating TGFβ/Smad functional activity, we then show that a novel V717M-WWP2 mutant in the MZ7-mel melanoma cell line forms a stable complex with the WWP2-N isoform and promotes EMT by stabilizing Smad3 protein levels. Finally, we report the first analysis of WWP2 expression in cancer cDNA panel arrays using WWP2 isoform-specific probes and identify unique patterns of WWP2 isoform abundance associated with early/advanced disease stages. WWP2-N is significantly downregulated in stage IIIC melanoma and up-regulated in stage II/III prostate cancer, and we also find isolated examples of WWP2-FL and WWP2-C overexpression in early-stage breast cancer. Together, these data suggest that individual WWP2 isoforms, and particularly WWP2-N, could play central roles in tumourigenesis linked to aberrant TGFβ-dependent signalling function, and also have potential as both prognostic markers and molecular therapeutic targets.

1. Introduction

Ubiquitination represents an important post-translational modification that controls protein turnover, activity and subcellular localization [1,2]. Ubiquitin is covalently attached to proteins via a three component enzymatic cascade involving its sequential transfer from a ubiquitin activating enzyme (E1) to a ubiquitin conjugating enzyme (E2), and then onto specific target substrates via a ubiquitin protein ligase (E3). There are around 600 E3 ligases in the genome [3], and WWP2 E3 ubiquitin ligase is one of nine related Nedd4 subfamily members, containing an N-terminal Ca^{2+}/phospholipid-binding C2 domain, four adjacent WW domains and a C-terminal HECT catalytic domain [4,5]. Interestingly, there are three separate WWP2 isoforms encoded within the WWP2 gene locus including full-length WWP2 (WWP2-FL), an N-terminal isoform lacking the HECT domain but incorporating a single WW1 domain, and a C-terminal isoform containing the HECT catalytic domain and WW4 domain [6,7].

Several WWP2 substrates associated with tumourigenesis have been identified and characterized including the PTEN tumour suppressor and key signalling components within the transforming growth factor-β (TGFβ) pathway known as Smads, and ectopic WWP2 overexpression also increased tumour cell spread in animal models [8,9]. Whilst TGFβ signalling serves a dual biological purpose in that it can cause cells to undergo cell cycle arrest and apoptosis, it also stimulates cells to undergo a differentiation process known as epithelial mesenchymal transition (EMT) that drives tumour cell dispersion and metastasis [10,11]. Interestingly, individual WWP2 isoforms have been shown to bind differentially to Smads, providing a novel regulatory mechanism that fine-tunes the levels of both activating and inhibitory Smads [9]. WWP2-FL selectively interacted with Smad2, Smad3 and Smad7 following TGFβ stimulation, and Smad7 is a preferred substrate. However, WWP2-N was also shown to form a complex with WWP2-FL in the absence of TGFβ, and dose-dependently activated WWP2-FL to drive rapid degradation of unstimulated Smad2 and Smad3. Following TGFβ stimulation, this WWP2-FL/WWP2-N complex rapidly dissociated, and subsequently switched WWP2-FL substrate specificity towards inhibitory Smad7. Thus, WWP2 isoforms have a unique ability to interchange Smad substrate specificity in the presence/absence of TGFβ, and dependent upon the expression of individual WWP2 isoforms.

In this study, and in light of their inter-dependent functional roles in oncogenic signalling mechanisms, we show that WWP2-N stable

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knockdown in carcinoma cells drives spontaneous induction of the EMT marker vimentin. Furthermore, we identify a mutant form of WWP2 found in the malignant MZ7-mel melanoma cell line that has altered specificity towards WWP2-N and R-Smads. The significance of WWP2-N as a key regulator of oncogenic TGFβ/Smad function is further highlighted when we quantitatively surveyed expression levels of individual WWP2 isoforms in cDNA arrays derived from breast cancer, prostate cancer and melanoma. Interestingly, we find that the levels of WWP2-N are significantly low in advanced relative to early stages of melanoma yet high in prostate cancer. Collectively, our data reveal important new insight into WWP2 isoform function linked to the tumourigenic role of Smad signalling that warrants further investigation of WWP2 ligase isoforms as novel cancer prognostic markers and therapeutic targets.

2. Materials and methods

2.1. Antibodies, DNA plasmids, reagents

Antibodies used for western blotting were: Rabbit anti-Smad3 (Chip grade, Abcam), Rabbit anti-Smad2 (Cell Signalling), Mouse anti-SMAD7 (New England Biolabs), mouse anti-human vimentin (Sigma), mouse anti-β actin (Sigma), and anti-WWP2-C and anti-WWP2-N (Abcam, UK), high affinity Rat anti-HA (Roche), and M2 Mouse anti-Flag (Sigma). Plasmids encoding the Flag-tagged derivatives of human WWP2, obtained from Fiona McDonald (University of Otago, NZ), were constructed by PCR cloning into pRK5 followed by the insertion of the Flag coding sequence at the 5′ terminal [9]. Derivation of human HA-/Flag-tagged Smad2, Smad3 and Smad7 is described elsewhere [12]. Human HA-tagged ubiquitin (expressed in pCDNA3.1+) was a kind gift from Dirk Bohmann (University of Rochester, USA). MG132 (Sigma) was reconstituted in DMSO and used at 10 μM, and SB431542 (Tocris Ltd) was used at 10 μM concentration. Recombinant TGFβ1 was from R&D systems.

2.2. Stable knockdown of WWP2 isoforms

Stable expression shRNA encoding plasmids specific for WWP2 isoforms were generated by annealing and cloning phosphorylated complementary oligonucleotides into Bgl II cleaved plasmid pTER, and used to generate stable Colo-357-Tet repressor expressing cells as described previously [9]. Expression was induced by stimulation of cells with 2 μM doxycycline (Melford Laboratories) for 48 h. Prior to lysis cells were washed in cold PBS, lysed in 1% v/v Igepal-630, 50 mM Tris pH 8.0, 150 mM NaCl, 10% v/v glycerol, 5 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4 and protease inhibitors (1% NP-40 LB), in the ratio 1 tablet (Roche):30 ml 1% NP-40LB. Lysates were mixed directly with SDS-PAGE Laemlli buffer (+10 mM DTT), and western blots developed using ECL reagent. The oligonucleotides used to generate WWP2 isoform shRNA plasmids are defined in Supplementary information.

2.3. Immunoprecipitation assays

Transfected cells treated with either 10 μM MG132 for 5 h in DMEM medium containing 2% FCS and treated +/− 5 ng/ml TGFβ for 1 h, washed in cold PBS, lysed in 1% v/v Igepal-630, 50 mM Tris pH 8.0, 150 mM NaCl, 10% v/v glycerol, 5 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4 and protease inhibitors (1% NP-40 LB), in the ratio 1 tablet (Roche):30 ml 1% NP-40LB. Lysates were cleared by centrifugation and incubated with 0.5 μg high affinity anti-HA antibody (Roche) and 20 μl of protein-G agarose (Sigma) overnight at 4 °C. Immune-complexes were harvested (2000 rpm; 30 s), repeatedly washed using 0.1% NP-40 LB, and resuspended in 15 μl Laemlli buffer (+10 mM DTT) followed by Western blotting analysis.

2.4. Cycloheximide chase experiments

HEK293 cells were transfected and after 48 h were pre-treated with 10 μM MG132 for 5 h in DMEM (+2% serum). Cycloheximide (20 μM) was added and cells incubated for 1 h. The medium was removed and replaced with DMEM medium (+2% serum) and 20 μg/ml cycloxeximide, and cells harvested at specified time-points. Cells were washed in ice cold PBS, lysed in 1% NP-40LB and analysed by Western blotting.

2.5. Luciferase reporter assays

The Smad3 reporter construct ($pCAGAC_{12}$-luc) and the Smad2-specific reporter plasmid DE-luc were kindly provided by Caroline Hill (CRUK Laboratories, UK). For each plasmid transfection, 250 ng $pCAGAC_{12}$-luc and 15 ng pRSV-β-galactosidase (pRSV-βgal) encoding plasmid were used in conjunction with Smad encoding plasmids as described previously [9].

2.6. Induction of EMT in Colo-357 cells

Colo-357 cells were seeded at 10^4 cells per 35 mm plastic well (Nunclon) and the next day left unstimulated, stimulated with 5 ng/ml TGFβ, 5 ng/ml TGFβ + SB431542, and everyday thereafter until day 6 (where the medium was changed every 2 days). Cells were then washed in PBS, lysed in 250 μl 1% NP40-LB to which 125 μl of 2× Laemlli buffer was added and samples boiled (10 min) and analysed by Western blotting.

2.7. Growth/survival assays

For growth/survival assays, 2×10^4 cells were seeded per well into 96 well plates and grown in tet approved serum overnight. Cells were grown for 48 h in the absence or presence of tet and serum starved for 16 h. Colo-357 cells were treated with TGFβ at 0.5 ng/ml for 48 h, or were left untreated. At the relevant time point, cell growth/survival was assessed using the CellTiter 96® One Solution Cell Proliferation Assay (Promega, UK).

2.8. WWP2 isoform expression analysis in primary tumour samples using real-time qRT-PCR analysis

For WWP2 isoform expression analysis in normal and cancer tissues, Tissue Scan™ tissue quantitative PCR arrays were used according to the manufacturer's protocol (Origene, Rockville, USA), and data normalised to β-actin. The array panels consisted of breast cancer cDNA derived from normal ($n = 7$), stage I ($n = 10$), stage IIA ($n = 13$), stage IIB ($n = 7$), stage IIIA ($n = 8$), stage IIIC ($n = 3$); melanoma cDNA from normal ($n = 3$), stage III ($n = 10$), stage IIIA ($n = 1$), stage IIIB ($n = 6$), stage IIIC ($n = 4$), stage IV ($n = 19$); prostate cancer cDNA from normal ($n = 7$), stage II ($n = 7$), stage III ($n = 3$), and non-recorded hyperplasia/prostatitis ($n = 31$). Quantitative qRT-PCR was performed as described previously [13]. The primer/probe sequences for detecting WWP2 isoform expression used were designed and manufactured by Primer Design, Southampton, UK (see Supplementary information for primer sequences). Cycling conditions were 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C repeated 40 times and 60 °C for 1 min. Reactions were performed using an ABI PRISM 7700 thermocycler (Applied Biosystems).

3. Results

3.1. WWP2 isoform depletion impacts differentially on the levels of Smads and the EMT marker vimentin

The WWP2 locus encodes three distinct isoforms including full-length WWP2 (WWP2-FL), an N-terminal isoform lacking the HECT

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