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Nuclear GSK-3β segregation in desmoid-type fibromatosis

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Aims: Desmoid-type fibromatosis (DF) is a rare benign myofibroblastic neoplasm of the connective tissue that is unable to metastasize but is associated with a high local recurrence rate. Nuclear β-catenin is the most commonly used histological marker of DF; however, clinical and biological predictive markers guiding the treatment and follow-up of DF are still lacking. Normally, β-catenin is regulated by the cytoplasmic multiprotein complex of adenomatous polyposis coli (APC), axin, casein kinase 1α (CK1α), and glycogen synthase kinase 3β (GSK-3β); this phosphorylates and degrades β-catenin, which would otherwise translocate to the nucleus. The aim of this study was to analyse the expression and localization of the β-catenin–protein complex of the Wnt pathway in cells isolated from DF patients.

Methods and results: We isolated cells from biopsies of DF patients, and demonstrated, by immunofluorescence and immunoblot analyses, that it is almost exclusively nuclear GSK-3β that colocalizes and interacts with β-catenin. The nuclear translocation of β-catenin and GSK-3β is not correlated with *CTNNB1* mutations. In DF samples, the multiprotein complex is disrupted, as the cytoplasmic localization of APC and axin makes interaction with the nuclear β-catenin and GSK-3β impossible.

Conclusions: Our data suggest that GSK-3β is an additional DF marker with an important role in the aetiopathogenesis of this entity.

Keywords: axin, cyclin D1, desmoid-type fibromatosis, GSK-3β, Wnt pathway, β-catenin

Introduction

Desmoid-type fibromatoses (DFs) are benign myofibroblastic tumours of the connective tissue that are locally invasive but lack metastatic potential.[1–3] DFs are rare tumours; they account for ~0.03% of all neoplasms and <3% of soft tissue tumours.[4] They can be diagnosed at any age, but are more common in young people (10–40 years), and are slightly more frequent in women than in men. Most DFs arise sporadically; some are related to pregnancy and trauma, and others appear as part of a syndrome with colonic polyposis, osteomas, and soft tissue tumours.[5,6]

DFs can develop in soft tissues at virtually any body site, but most commonly the abdominal wall, extremities, shoulder, neck, and chest wall.[4] Histological and cytological analyses reveal that DF cells are strongly positive for vimentin, calponin, and caldesmon; they show variable signals for smooth muscle actin and desmin, and are negative for E-cadherin and CD34.[4,7–9] Cytogenetic analyses show loss of 6q or 5q, trisomies 8 or 20, or monosomy 20, in 25–30% of cases.[10,11]

Most DFs show oncogenic β-catenin gene (*CTNNB1*) mutations; 70–80% of sporadic DFs have mutations in codons 41 or 45 of exon 3 of

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CTNNB1.[12,13] β-Catenin, a multifunctional protein, is required for cell adhesion, and has a function in transducing the Wnt signal from the cell surface to the nucleus.[14] In the absence of the Wnt signal, cytoplasmic β-catenin is regulated by a phosphorylation-degradation cascade mediated by two sequential events: casein kinase 1α (CK1α) phosphorylates Ser45 of β-catenin in order to prime glycogen synthase kinase 3β (GSK-3β) phosphorylation.[15–18]

GSK-3β is a multifunctional serine/threonine kinase involved in different cellular processes, and it is an important regulator of the Wnt–β-catenin signalling pathway. When β-catenin is present at high levels, GSK-3β binds the adenomatous polyposis coli (APC) protein, and the resulting multiprotein complex leads to β-catenin degradation. Whenever Wnt signalling inactivates GSK-3β, it prevents it from phosphorylating and degrading β-catenin, leading to the stabilization of β-catenin, which translocates to the nucleus.[19–21] A minority of apparently sporadic DFs have germ-line *APC* mutations.[1] In the presence of *APC* or *CTNNB1* mutations, the β-catenin multiprotein complex cannot be formed; therefore, β-catenin is not degraded, but accumulates in the nucleus and activates Wnt target gene expression.[14]

Dysregulation of GSK-3β has been implicated in tumorigenesis and cancer progression.[22] β-Catenin dysregulation is important in DF; indeed, nuclear β-catenin immunostaining is commonly used in the diagnosis of DF.[8,23] However, the choice of the optimal DF therapy is still difficult, because the diagnosis is rare, and the growth, stabilization and regression of DF are highly variable.

Here, we studied the β-catenin–protein complex of the Wnt pathway in cells isolated from DF patients with non-mutated or mutated β-catenin. We analysed total and phosphorylated β-catenin, and GSK-3β expression and distribution, and correlated them with *CTNNB1* exon 3 mutations. In DF cells, β-catenin is not the sole component of the complex accumulating in the nucleus; the enzyme GSK-3β is exclusively nuclear and is associated with β-catenin, and its nuclear localization is independent of β-catenin phosphorylation.

Materials and methods

BIOPSY SAMPLES

We collected fresh biopsies from six patients (samples 1–6) with sporadic DF in a sterile environment (during therapeutic surgery). Additionally, we collected normal fascia from DF patients and myofibroblastic tissue from non-desmoid individuals (seven samples) as controls. Samples from each patient were collected according to a specific standard operative procedure approved by the Veneto Institute of Oncology and the University of Padua; informed consent for research use was obtained from each patient.

Three samples were from males (aged 52, 53 and 53 years) and three were from females (aged 31, 40 and 41 years). All lesions involved the extremities (arm, shoulder, or leg), except for sample 3, where the lesion was localized in the abdominal wall.

DESMOID-TYPE FIBROMATOSIS CELL CULTURES

Fresh biopsy tissues were mechanically fragmented, seeded in 12-well cell culture plates, and cultured in 1 ml of CHANG medium [18% Chang B and 2% Chang C (Irvine Scientific, Santa Ana, CA, USA) diluted in Alpha MEM medium, (Gibco, Life Technology Grand Island, NY, USA)] with 50% heat-inactivated fetal bovine serum (FBS) and antibiotics (Gibco), for 1 week at 37°C in 5% CO_2, without replacing the medium. Then, the primary DF cells were grown as a monolayer in CHANG medium with 10% FBS and antibiotics. The cells used in the following experiments were obtained from four or five culture passages.

GENOMIC DNA ISOLATION AND *CTNNB1* SEQUENCING

Fifty nanograms of genomic DNA, extracted from DF primary cell cultures by the salting-out method, were used for sequencing the exonic structure of *CTNNB1* (NM_001098210). The PCR fragments (primer sequences are available on request) were labelled using the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Life Technology, Grand Island, NY, USA), purified through fine columns (Sephadex G50; GE Healthcare Life Science, Waukesha, Wisconsin USA), and resolved in an ABI PRISM Sequencer 3130 (Applied Biosystems).

SINGLE AND DOUBLE IMMUNOFLUORESCENCE LABELLING

The cells were grown to 40–60% confluence on sterile glass coverslips, fixed with 4% paraformaldehyde, permeabilized with ice-cold methanol, washed once with PBS (137 mM NaC1, 2.7 mM KC1, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), and saturated with 5% bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, Missouri, USA). They were then incubated overnight at 4°C with primary antibody, followed by fluorescent secondary antibody, both diluted in 1% BSA/

PBS; this was followed by washes with PBS. Finally, they were fixed with mounting medium containing DAPI (Vectashield; Vector Laboratories, Burlingame, California, USA). For double immunofluorescence labelling, the two primary antibodies were incubated together, as were the two secondary antibodies, followed by extensive washing with PBS after incubation.

The primary antibodies were as follows: anti-β-catenin, clone 6F9 (Sigma-Aldrich; 1:1000), anti-phospho-β-catenin (Thr41/Ser45; Cell Signaling, Danvers, MA, USA; 1:100), anti-APC (F-3; Santa Cruz, Dallas, Texas U.S.A.; 1:100), anti-GSK-3β (H-76; Santa Cruz; 1:100), and anti-axin1 (C76H11; Cell Signaling; 1:50). The following secondary antibodies were from Molecular Probes, Life Technology Grand Island, NY, USA: Alexa-Fluor 488-conjugated goat anti-mouse IgG (1:200) and Alexa-Fluor 568-conjugated goat anti-rabbit IgG (1:400).

Slides were analysed using an Olympus Provis AX70 fluorescence microscope. Images were recorded with a COHO High Performance CCD Camera, and processed with MACPROBE v4.3.

WHOLE CELL EXTRACT PREPARATION AND WESTERN BLOT ANALYSIS

The harvested cells were incubated with lysis buffer (20 mM Hepes, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) for 30 min on ice. Lysates were centrifuged at 13 300 ***g*** for 10 min at 4°C. Equal amounts of whole-cell extract (100 μg), determined by the BCA Assay (Sigma-Aldrich), were separated by 12.5% SDS-PAGE and transferred to a PVDF membrane (BioRad, Hercules, CA, USA). Non-specific proteins were blocked with 5% (w/v) BSA in TBS (150 mM NaCl, 10 mM Tris–HCl, pH 8.00). The membrane was then probed with the primary antibodies diluted in 5% BSA/TBST (TBS with 0.01% Tween-20), and, after washes with TBST, incubated with the peroxidase-conjugated secondary antibodies, diluted in TBST [anti-mouse IgG, 1:80000 (Sigma, Saint Louis, Missouri, USA); anti-rabbit IgG, 1:20000 (Millipore, Billerica, MA, USA)]. The signals were detected using an enhanced chemiluminescence kit (Thermo Scientific, Waltham, MA, USA). We used the following primary antibodies from Sigma-Aldrich: anti-β-catenin (1:500), anti-phospho-β-catenin, Thr41/Ser45 (1:100), and anti-β-actin (clone AC-74, 1:2000).

ISOLATION OF NUCLEAR AND CYTOPLASMIC PROTEINS

Cellular fractionation was performed as described by Abmayr and modified by Li.[24,25] One hundred micrograms of nuclear and cytoplasmic protein fractions, separated by SDS-PAGE and immunoblotted, were incubated overnight at 4°C with the appropriate dilutions of antibodies specific for β-catenin, GSK-3β, and GAPDH (MAB374, 1:500; Millipore). The blots were then incubated with the secondary antibodies, and developed as described above.

COIMMUNOPRECIPITATION OF β-CATENIN AND GSK-3β

Three hundred micrograms of total proteins or nuclear fractions were extracted with TEN buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, and protease cocktail). The lysates were incubated with 1–2 μg of anti-β-catenin antibody with gentle shaking at 4°C overnight. The antibody was recovered using rProtein Agarose G, separated by SDS-PAGE, and developed with anti-GSK-3β antibody.

RNA EXTRACTION AND REAL TIME-PCR

Total RNAs were isolated from desmoid or non-desmoid cells (controls) after trypsinization with TRIzol solution (Invitrogen, Life Technology Grand Island, NY, USA), according to the manufacturer's instructions. RNA was quantified by spectrophotometric analysis (NanoVue; GE Healthcare). Equal amounts of RNA (500 ng) were treated with DNase I, and cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). The cDNA concentrations for genes encoding axin2, c-myc and cyclin D1 were quantified by real-time PCR using iQ SYBR Green Supermix (Invitrogen), with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The PP1A gene was used as a housekeeping gene. The sequences of the primers are given in Table 1. Data analysis was performed using the second derivative method algorithm.

CELL PROLIFERATION ASSAY

DF and non-desmoid cells were seeded at 1500 cells/cm^2 in 96-well culture plates in triplicate, and incubated at 37°C in 5% CO_2. The medium was replaced every second day, and, after 1 week, cell proliferation was evaluated using the MTT assay (Promega, Madison, WI, USA). The absorbance after incubation with MTT for 2 h at 37°C in 5% CO_2 was measured with an enzyme-linked immunosorbent assay (ELISA) plate reader. The viable cell number was determined by reading the optical density (OD) value at 490 nm.

Table 1. Sequences of primers used in real-time PCR

Primers	Sequences
PP1A	F: 5′-TGGGCCGCGTCTCCTTTGAG-3′
	R: 5′-ACCAAATCCTTTCTCTCCAGTGCTC-3′
Axin2	F: 5′-AGCCAAAGCGATCTACAAAAGG-3′
	R: 5′-GGTAGGCATTTTCCTCCATCAC-3′
c-myc	F: 5′-TCAAGAGGCGAACACACAAC-3′
	R: 5′-GGCCTTTTCATTGTTTTCCA-3′
Cyclin D1	F: 5′-GTGCTGCGAAGTGGAAACC-3′
	R: 5′-ATCCAGGTGGCGACGATCT-3′

Results

β-CATENIN AND GSK-3β ARE ASSOCIATED IN THE NUCLEI OF DESMOID-TYPE FIBROMATOSIS CELLS

We isolated primary cells from surgical biopsies from patients with DF and from surgical biopsies of connective tissue of non-desmoid individuals (controls). Cells originating from DFs were processed for *CTNNB1* sequencing, which identified two apparently heterozygous mutations in exon 3: c.133C>T (p.Ser45Phe) in samples 1 and 4; and c.121A>G (p.Thr41Ala) in sample 5. Samples 2, 3 and 6 did not show any mutations of β-catenin. All patients had sporadic DF, with the exception of patient 2, who had local recurrence of DF; none of them had polyposis.

To investigate the involvement of the Wnt pathway in DF, the cells were initially immunostained with antibodies specific for β-catenin and GSK-3β. Immunofluorescence data showed that, in DF cells, both β-catenin and GSK-3β were translocated to the nucleus and colocalized, as seen by the yellow signal in the merged images (Figure 1A). A fraction of β-catenin was also localized in the cell membrane. We quantified the cells with nuclear positivity for β-catenin and GSK-3β, by counting 100 cells in several randomly chosen fields. The average proportion of nuclear GSK-3β-positive cells was 92.4%, and the average proportion of nuclear β-catenin-positive cells was 81.7% (Table 2); all of the nuclear β-catenin colocalized with the nuclear GSK-3β. Moreover, whereas the intensity of the nuclear β-catenin signal was different between cells carrying and not carrying the *CTNNB1* mutation, varying from a high to a low level, the GSK-3β nuclear signal was strongin mutated and non-mutated DF samples (Figures 1A and S1).

The nuclear localization of both β-catenin and GSK-3β was also investigated by immunoblotting of nuclear and cytoplasmic proteins extracted from DF cells. Non-desmoid samples were processed in parallel, as controls (Figure 1B). The immunoblotting data showed intense β-catenin and GSK-3β signals in the nuclear fraction, whereas there was a barely detectable, very faint band in the cytoplasmic fraction in some samples (Figure 1B), confirming the nuclear translocation of β-catenin and GSK-3β in DF. Whereas GSK-3β was absent in the nuclear fraction of non-desmoid samples, a proportion of nuclear β-catenin was also detected in non-desmoid cells (Figure 1B). Immunoblotting of GAPDH in the cytoplasmic and nuclear fractions of non-desmoid and desmoid samples demonstrated a low level of contamination of cytoplasmic proteins in the nuclear fraction (Figure 1C).

We also performed immunoprecipitation assays with nuclear and whole cell lysates, in order to detect the interaction between β-catenin and GSK-3β. Our results demonstrated that not only did β-catenin and GSK-3β colocalize in the nucleus, but, most importantly, they were associated, forming a nuclear complex (Figure 1D). In contrast, β-catenin and GSK-3β did not associate in the nuclei of control cells (Figure 1D).

PHOSPHORYLATED β-CATENIN IS ABSENT IN DESMOID-TYPE FIBROMATOSIS CELLS

As described above, two individuals had a mutation of *CTNNB1* in Ser45 (cases 1 and 4) and one had a mutation in Thr41 (case 5); both are sites of β-catenin phosphorylation, and their impairment blocks this process.

Therefore, we investigated the phosphorylation level of β-catenin in DF cells with immunofluorescence and immunoblotting. DF cells showed a very weak cytoplasmic Thr41/Ser45 phospho-β-catenin signal in mutated and non-mutated samples (Figure 2A). Conversely, we detected phospho-β-catenin in non-desmoid cells (Figure 2A). Immunoblotting data confirmed the absence of phosphorylated β-catenin in DF cells, in contrast to the presence of phospho-β-catenin in the control sample. Protein levels were normalized to β-actin (Figure 2B). However, the level of total β-catenin was higher in DF samples than in control samples, as detected by quantification of the β-catenin immunoblot, normalized to β-actin (Figure 2C).

In summary, we determined that DF cytoplasmic β-catenin is not phosphorylated, and consequently is

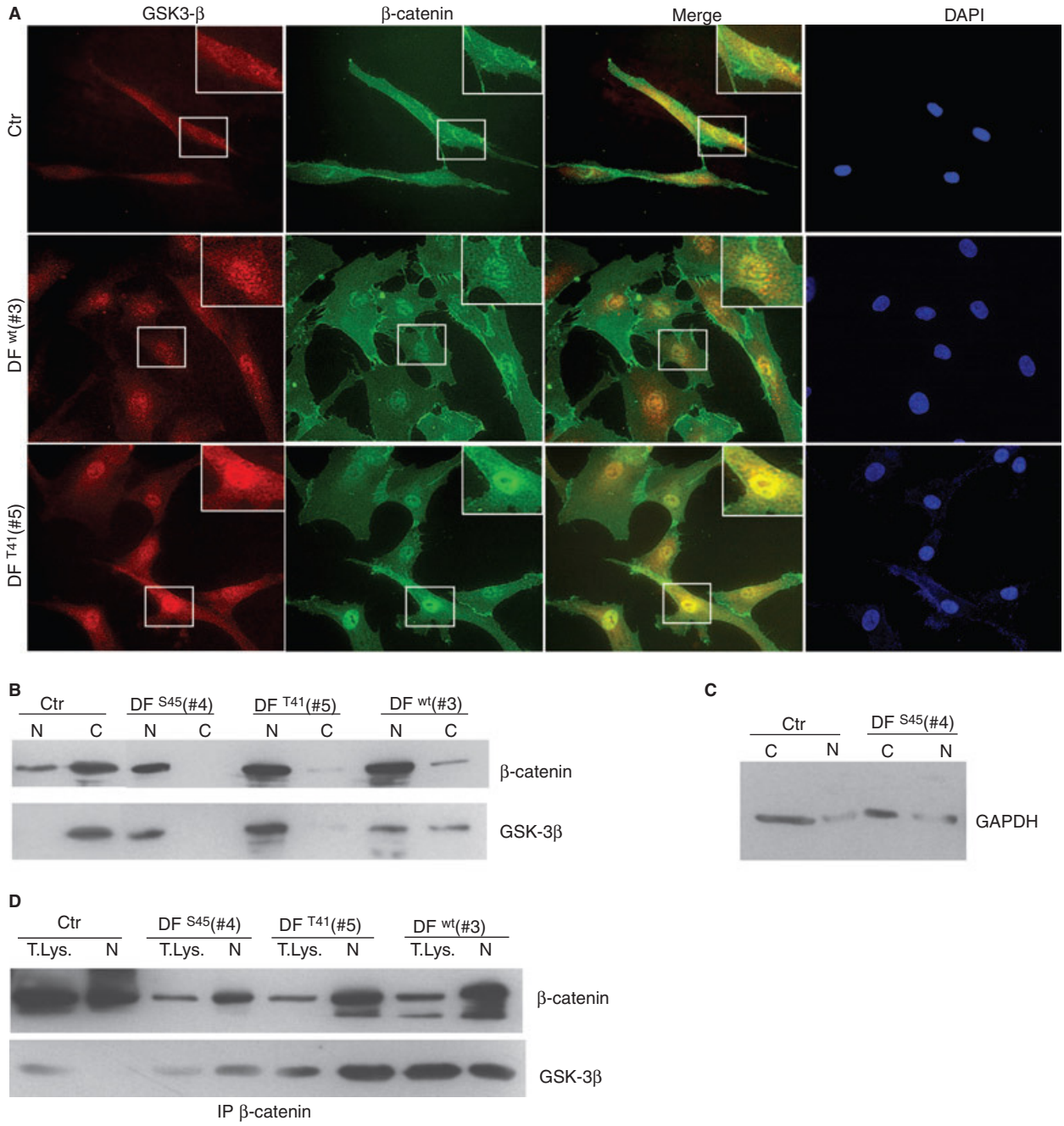

Figure 1. Nuclear colocalization of GSK-3β and β-catenin in desmoid-type fibromatosis (DF) cells. **A,** DF cells (samples 3^{WT} and 5^{T41}) and control cells (ctr) were immunostained with anti-β-catenin (green) and anti-GSK-3β (red) antibodies. The nucleus was stained with DAPI (blue). The merged picture shows colocalization of β-catenin and GSK-3β (yellow spots). Inset: magnification of the boxed cells. The pictures show the nuclear localization of β-catenin and GSK-3β in DF cells, and cytoplasmic staining in control cells. **B,** Immunoblotting of nuclear (N) and cytoplasmic (C) DF cell extracts. Fifty micrograms of nuclear and cytoplasmic proteins of the DF samples (3^{WT}, 4^{S45}, and 5^{T41}) were subjected to SDS-PAGE followed by immunoblotting with anti-β-catenin and anti-GSK-3β antibodies. There were high levels of β-catenin and GSK-3β in DF cell nuclei, whereas, in the cytoplasm, β-catenin and GSK-3β were absent or only weakly visible. Cytoplasmic β-catenin and GSK-3β were more highly expressed in control cells than in DF cells. **C,** Immunoblotting of nuclear (N) and cytoplasmic (C) DF cell and control cell extracts with anti-GAPDH antibody. GAPDH was found in the cytoplasmic fraction, and a trace was found in the nuclear fraction. **D,** Colocalization of β-catenin and GSK-3β as demonstrated by immunoprecipitation experiments. Three hundred micrograms of total cell extract (T. Lys.) and nuclear (N) fractions of DF cells were immunoprecipitated with β-catenin, and immunoblotted with anti-GSK-3β antibody. The results showed that β-catenin and GSK-3β interact with each other. A cell extract from non-desmoid cells was used as the control (ctr).

Table 2. Cells with prevalent nuclear or cytoplasmic localization of β-catenin and GSK-3β; for all samples, 100 cells were counted and divided according to the ratio between the nuclear and cytoplasmic signals

	β-Catenin		GSK-3β	
Samples	Nucleus (%)	Cytoplasm (%)	Nucleus (%)	Cytoplasm (%)
1^{S45}	88	12	97	3
2^{WT}	80	20	95	5
3^{WT}	72.7	27.3	90	10
4^{S45}	84	16	85	15
5^{T41}	84	16	95	5
Control	0	100	0	100

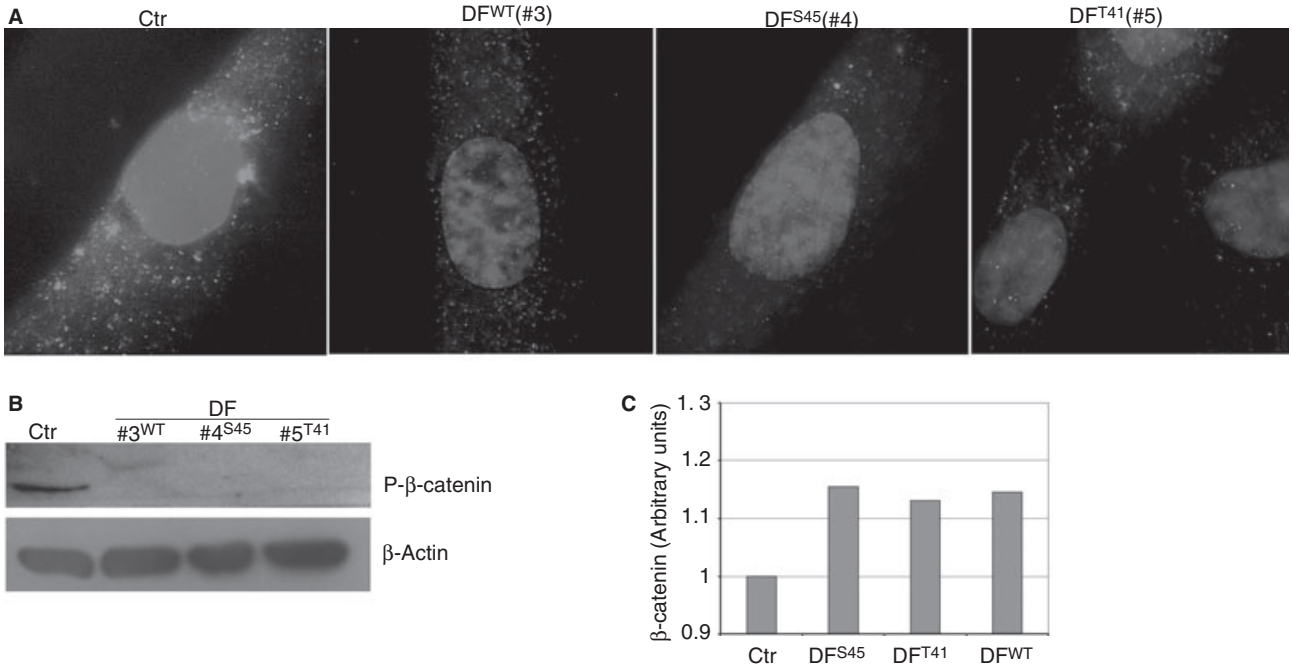

Figure 2. Phosphorylation of β-catenin in desmoid-type fibromatosis (DF) cells. **A**, DF cells (samples 3^{WT}, 4^{S45}, and 5^{T41}) and control cells (ctr) were immunostained with anti-phospho-(Thr41/Ser45)-β-catenin antibody (see spots in the cytoplasm). The cell nuclei were stained with DAPI. The pictures show the absence or scarcity of a phospho-β-catenin cytoplasmic signal in DF samples as compared with control cells. **B**, Immunoblotting of a total cell extract of DF cells as compared with control cells. Equal amounts of protein extract (100 mg) were subjected to SDS-PAGE followed by immunoblot analysis with anti-phospho-β-catenin antibody. Note the absence of a phospho-β-catenin signal in DF cells, and the presence of a signal in the control cells. Cell extracts were normalized with β-actin. **C**, Graph of the total β-catenin level in non-desmoid (ctr) and desmoid (DF) whole protein extract, normalized to β-actin.

not degraded, resulting in its nuclear accumulation (Figures 1 and 2).

APC AND AXIN ARE UNABLE TO INTERACT WITH β-CATENIN OR GSK-3β

The regulation of β-catenin occurs via a cytoplasmic multiprotein complex that includes GSK-3β, CK1α, axin, and APC. To further investigate the almost exclusively nuclear localization of GSK-3β and β-catenin, we extended the studies to APC and axin, which are known to be critical for β-catenin regulation.[18,20,26]

For this purpose, we performed double immunofluorescence labelling in DF and control cells with antibodies against APC and axin. The results

demonstrated that APC and axin are colocalized in the cytoplasmic compartment, as shown by the yellow signal in the merged images (Figure 3A), with only a faint nuclear signal for APC.

Furthermore, the different subcellular distribution of axin/β-catenin and APC/GSK-3β was confirmed by double immunofluorescence labelling. These results demonstrated the different localization of Wnt pathway proteins, further showing that the multiprotein complex that is responsible for β-catenin phosphorylation cannot be assembled (Figure 3B,C).

THE TRANSCRIPTION GENES ENCODING AXIN2 AND CYCLIN D1 ARE DIFFERENTIALLY REGULATED IN DF CELLS

In order to investigate the potential alteration of expression of the transcriptional targets of the Wnt pathway, we analysed the mRNA levels of *AXIN2*, *c-myc* and *CCND1* in DF samples. For this purpose, we performed real-time PCR, comparing DF samples with non-desmoid samples. The results demonstrated that *c-myc* was not expressed in DF cells, whereas the

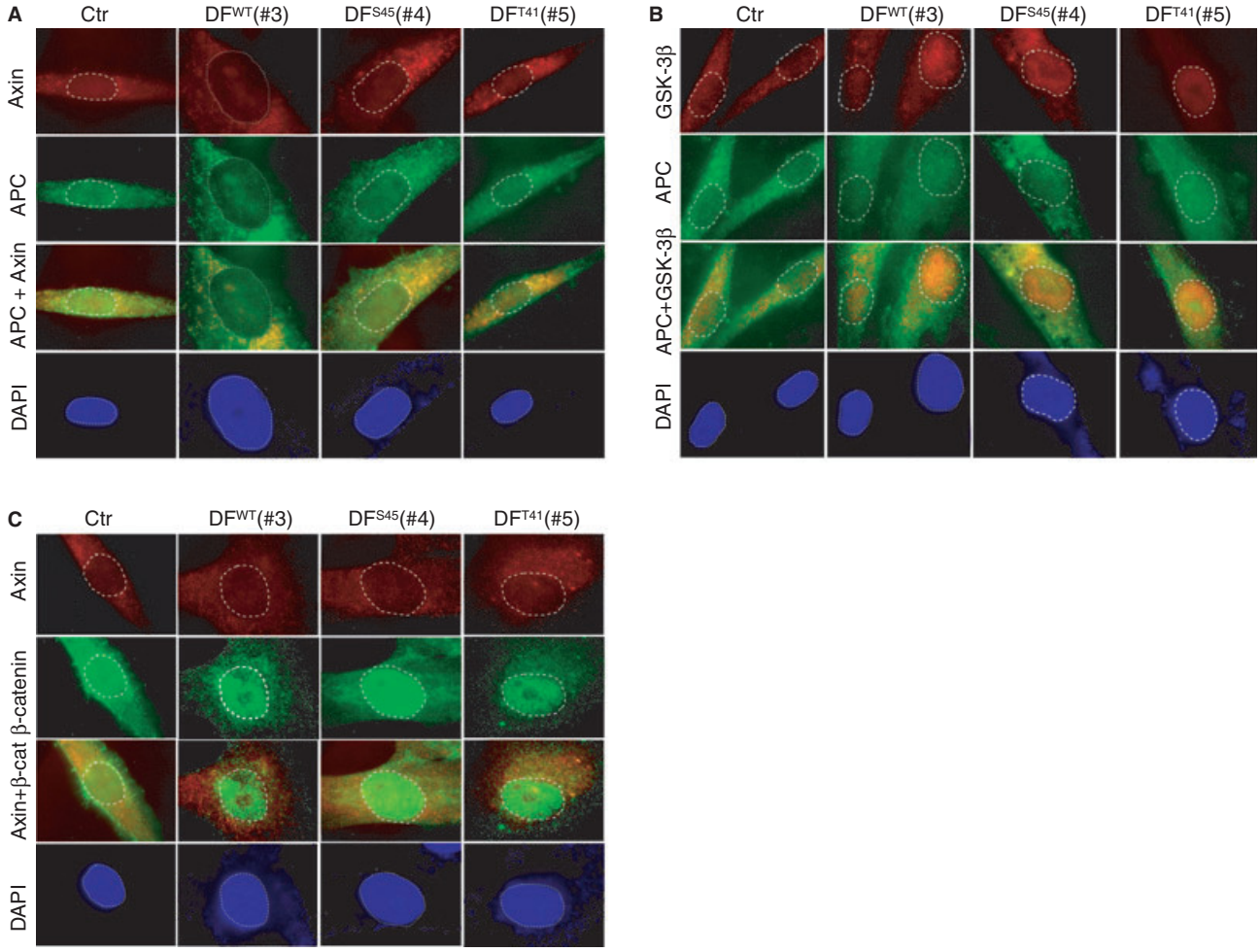

Figure 3. Localization of proteins of the Wnt pathway in DF cells. **A,** Immunofluorescence of APC and axin in DF cells. DF cells (samples 3^{WT}, 4^{S45}, 5^{T41}) and control cells (ctr) were immunostained with anti-APC (green) and anti-axin (red) antibodies. The cell nuclei were stained with DAPI (blue). APC and axin were distributed in the cytoplasmic compartment and mostly colocalized (yellow spots in the merged picture). **B,** Immunofluorescence of APC and GSK-3β in DF cells. DF cells (samples 3^{WT}, 4^{S45}, and 5^{T41}) and control cells (ctr) were immunostained with anti-APC (green) and anti-GSK-3β (red) antibodies. The cell nuclei were stained with DAPI (blue). The picture shows GSK-3β localization in the nuclei of DF cells, whereas APC showed a diffuse signal in the cytoplasm and a weak signal in the nucleus. Non-desmoid cells (ctr) showed cytoplasmic distribution of both APC and GSK-3β. **C,** Immunofluorescence of β-catenin and axin in DF cells. DF cells (samples 3^{WT}, 4^{S45}, and 5^{T41}) and control cells (ctr) were immunostained with anti-β-catenin (green) and anti-axin (red) antibodies. The cell nuclei were stained with DAPI (blue). The picture shows β-catenin localization in the nuclei of DF cells, and localization in the cytoplasm in control cells. Axin was localized in the cytoplasm in both DF and control cells.

expression of *AXIN2* was increased two-fold to six-fold in DF samples as compared with controls, with the exception of sample 3. In contrast, the cyclin D1 gene was downregulated in DF cells, with a two-fold to four-fold reduction in cyclin D1 mRNA expression in DF samples as compared with controls (Figure 4A).

In order to investigate the effect of cyclin D1 on cell proliferation, we performed an MTT assay to compare cell growth between DF and control cells. The results showed diminished cell growth of DF cells as compared with non-desmoid cells, suggesting a relationship between the decrease in cyclin D1 expression and the reduction in cell proliferation (Figure 4B).

Discussion

In this study, we analysed the expression and localization of the protein complex of the Wnt pathway in myofibroblastic primary cells isolated from surgical specimens of DF. Cells isolated from the margins and from the central portions of the biopsy samples were morphologically very similar, and had similar growth rates; however, cell size was extremely heterogeneous between samples and even within the same sample (Figure 3). *CTNNB1* genotyping of the samples demonstrated that three individuals did not carry mutations, whereas three carried mutations of exon 3, two at Ser45 and one at Thr41.

β-Catenin activity is commonly regulated by a cytoplasmic multiprotein complex that includes axin, APC, CK1α, and GSK-3β, which sequentially phosphorylate β-catenin, marking it for degradation through the ubiquitin–proteasome system, maintaining a low cytoplasmic level of β-catenin.[15,16,20] When the β-catenin phosphorylation/degradation process fails, it causes an elevation in the level of free cytoplasmic β-catenin, leading to its translocation to the nucleus.[16,19] Nuclear β-catenin is consistently observed in aggressive fibromatosis, and it has also been frequently found during the progression of several tumours.[12,21,27] Our findings confirm that β-catenin is mostly translocated to the nucleus with a slight difference in nucleus-positive cells between *CTNNB1*-mutated and non-mutated samples (mutated, 85.3%; non-mutated, 76.35%; absent in negative controls). Cell fractionation confirmed the predominance of nuclear β-catenin of DF samples independently of the *CTNNB1* mutations. Traces of cytoplasmic β-catenin were detected in some DF samples, but it is impossible to determine whether this resulted only from contamination during the nuclear/cytoplasmic fractionation.

The novelty of our results is the impressive GSK-3β nuclear signal in all of the DF cells, with equivalent

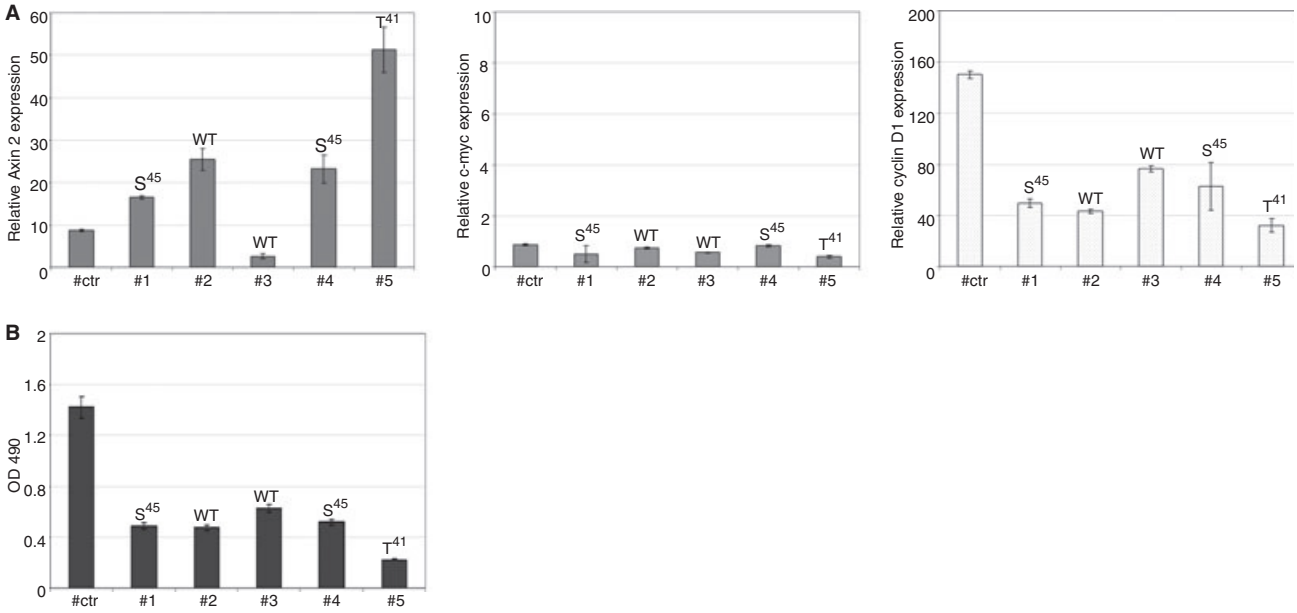

Figure 4. Expression of axin2, c-myc and cyclin D1 in desmoid-type fibromatosis (DF) cells and DF cell proliferation. **A,** Total RNA was extracted from DF and non-desmoid cells (ctr). Axin2, c-myc and cyclin D1 expression was analysed by real-time PCR. Expression level is depicted as *n*-fold of the normalized amount of mRNA from untreated cells [1 AU = mRNA gene concentration (fmol/μl)/mRNA PP1A (fmol/μl)] of triplicate reactions for each sample. **B,** Equal numbers of DF and control cells were plated on 96-well plates in triplicate. Cells were cultured for 1 week, and the viable DF cell number was then determined with the colorimetric MTT assay.

percentages of nucleus-positive cells in mutated and non-mutated DF samples (mutated, 92.3%; non-mutated, 92.5%; absent in negative controls), suggesting that the nuclear GSK-3β translocation is not directly correlated with *CTNNB1* mutations. The higher percentage of nuclear GSK-3β-positive cells and the intensity of the signal suggest that nuclear GSK-3β could be an additional marker for DF cells, alongside nuclear β-catenin.

It is known that GSK-3β is essential for many signalling pathways and cellular processes; a malfunction of this kinase is implicated in the pathogenesis of a number of diseases, including diabetes, bipolar disorder, Alzheimer's disease, heart failure, and cancer.[22,28,29] GSK-3β has a key role in the regulation of the cytoplasmic level of β-catenin; it is required for the cascade of β-catenin phosphorylation at Thr41, Ser37, and Ser33, after the phosphorylation of Ser45 by CK1α.[15,16] Nuclear GSK-3β has an additional subcellular role in controlling Wnt signalling levels by a mechanism that does not involve β-catenin phosphorylation, but GSK-3β–axin binding.[30]

Thus, although GSK-3β is part of the cytoplasmic β-catenin degradation process, here we have demonstrated that, in DF cells, GSK-3β enters the nucleus and is found in a complex with β-catenin; the absence of phosphorylated β-catenin indicates that the mechanism does not involve successful β-catenin phosphorylation.[15,16]

In the canonical Wnt–β-catenin pathway, besides the kinases CK1α and GSK-3β, the two scaffold proteins APC and axin have key roles in the formation of the degradation complex. In the inactivated state of the Wnt pathway, axin acts as a scaffold for the other proteins, CK1α and GSK-3β phosphorylate β-catenin, and the entire process is accelerated by APC. Upon Wnt activation, the Wnt ligands might regulate GSK-3β by disrupting the interaction between APC and the axin–GSK-3β complex. The dissociation of APC from axin results in a reduction of GSK-3β activity and the activation of Wnt downstream signalling.[20,31] Here, we have demonstrated cytoplasmic localization of APC and axin and nuclear translocation of β-catenin and GSK-3β, which leads to disassembly of the multiprotein complex and thus the absence of the β-catenin phosphorylation/degradation process.

The phosphorylation by GSK-3β of primed β-catenin (already phosphorylated by CK1α) is dependent on the interaction between β-catenin and the other proteins of the complex, such as axin and APC. Therefore, in the absence of these proteins, the cytoplasmic β-catenin complex cannot be assembled, and the phosphorylation cannot take place. Both in the case of *CTNNB1* mutations and the case of defects of APC (or axin), β-catenin escapes degradation: in the first case, because GSK-3β binds to an altered β-catenin that cannot be phosphorylated; and in the second case, because GSK-3β binds to β-catenin but seems unable to phosphorylate it in the absence of the formed multiprotein complex. Therefore, in both cases, GSK-3β binds to β-catenin but the phosphorylation process cannot take place. In other words, the pathogenesis starts with different mechanisms, but converges in the nuclear migration of the β-catenin–GSK-3β complex, owing to the lack of β-catenin phosphorylation/degradation.

It is known from the literature and previous studies that, besides β-catenin, other components of the β-catenin degradation complex are implicated in DF. Our data clearly indicate that GSK-3β is one of the key components with an important role in aggressive fibromatosis.

Aberrant stabilization of β-catenin triggers up-regulation of Wnt target genes, such as those encoding c-myc and cyclin D1, and is believed to be the basis for tumorigenesis.[26,32] Here we demonstrated that, whereas *c-myc* was not expressed in DF samples as in normal samples, *AXIN2* was up-regulated in DF samples, with the exception of the only sample derived from the abdominal fibromatosis. Moreover, in contrast to previous studies demonstrating overexpression of cyclin D1 in 59% of immunostained desmoid tissues,[33] we showed that cyclin D1 mRNA was down-regulated in all of the DF samples. It has been reported that GSK-3β is involved in the regulation of cyclin D1 mRNA transcription and protein degradation.[34] We believe that, in DF, GSK-3β might also play a role in cyclin D1 degradation.

Our results provide new perspectives on the possible molecular events of DF: (i) GSK-3β translocates to the nucleus independently of the presence of *CTNNB1* mutations, suggesting that other mechanisms involving the multiprotein complex might be involved; (ii) the axin–CK1α–GSK-3β–APC protein complex cannot assemble, as the cytosolic GSK-3β is absent; (iii) β-catenin is not phosphorylated, because the axin–CK1α–GSK-3β–APC protein complex is not formed, and not only because of mutations in *CTNNB1*; and (iv) GSK-3β appears to have additional functions in the nucleus, e.g. the regulation of cyclin D1.

Future work will focus on the nuclear function of GSK-3β and the interactions of the proteins in the Wnt pathway. An extended study is underway to validate the viability of nuclear GSK-3β as a pathognomonic DF marker.

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Conflict of interest

The authors do not have any conflicts of interest. The research was performed with internal funding and the aid of the Desmon association.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Nuclear localization of β-catenin in desmoid-type fibromatosis (DF) cells. DF cells (#1^{S45}; #2^{WT}; #4^{S45}) and control cells (ctr) were immunostained with β-catenin antibody (green). Nuclei were stained with DAPI (blue). Insets show magnification of the boxed cells.

