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Beta-catenin expression in Dupuytren's disease: potential role for cell–matrix interactions in modulating beta-catenin levels *in vivo* and *in vitro*

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Dupuytren's disease (DD) is a superficial fibromatosis of the hand. Although the molecular mechanisms responsible for this disease are unknown, recent studies suggest that beta-catenin may be a key factor involved in fibromatosis. In this study, we analysed the *in vivo* and *in vitro* expression levels of beta-catenin in DD, using surgical specimens and primary cell lines. Although no somatic mutations (exon 3) of beta-catenin were detected, Western blot analysis revealed high levels of beta-catenin in diseased palmar fascia, and low to undetectable levels of beta-catenin in patient-matched normal palmar fascia. Immunohistochemistry analysis showed high levels of beta-catenin expression within the disease fascia, as well as cytoplasmic and nuclear accumulations of the protein. Immunoprecipitation of beta-catenin from seven patient lesions showed the protein to be tyrosine phosphorylated. Lastly, Western analysis of three patient-matched (disease and normal fascia) primary cell cultures showed significantly elevated levels of beta-catenin in disease cells cultured in three-dimensional collagen lattices. This is the first extensive *in vivo* and *in vitro* characterization of beta-catenin in DD, and the first to suggest that the extracellular matrix may play an important role in modulating beta-catenin stability in DD.
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Dupuytren's contracture or disease (DD) is a benign, but debilitating fibroproliferative disease of the palmar fascia (Dupuytren, 1834) that causes fixed, flexed position of the fingers (Rayan, 1999). Clinically, DD is classified as a superficial fibromatosis that progresses through distinct clinical stages. The earliest stage of the disease is characterized by the appearance of small nodules of hyperproliferative cells that, over time, gives rise to collagen-rich disease cords. Although a number of risk factors have been proposed for DD, including alcoholism (Noble *et al*., 1992; Burge *et al*., 1997), trauma (Skoog, 1960; Mikkelsen, 1978; McFarlane, 1991), diabetes (Noble *et al*., 1984), smoking (An *et al*., 1988; Burge *et al*., 1997), and epilepsy (Arafa *et al*., 1992), their exact role in the disease is unclear (Ross, 1999).

Despite its classification as a benign disease, DD does display several features of a cancer. Firstly, there is strong evidence to suggest that DD is a genetic disease (Burge, 1999). This is supported by the appearance of distinct chromosomal abnormalities when cells from lesions are cultured *in vitro* (Sergovich *et al*., 1983; Wurster-Hill *et al*., 1988; Bonnici *et al*., 1992; Dal Cin *et al*., 1999; De Wever *et al*., 2000). Secondly, like most cancers, DD has multiple clinical stages, with the earliest stages of the disease being characterized by the appearance of small nodules of hyperproliferative cells (Kloen, 1999). Thirdly, DD has a high rate of recurrence following surgery and can display an invasive phenotype, infiltrating local tissues such as the skin. Lastly, recent epidemiological studies have shown increases in total mortality and cancer mortality among men with established DD, even after adjustment for age, smoking, and other confounding variables (Mikkelsen *et al*., 1999; Gudmundsson *et al*., 2002). Altogether, these findings suggest that DD and cancers may have more in common than previously appreciated, including perhaps similar pathomechanisms.

A recent immunohistochemistry (IHC) study has documented beta-catenin expression in several types of superficial fibromatoses, including DD (Montgomery *et al*., 2001). However, unlike more aggressive fibromatoses (Tejpar *et al*., 1999; Abraham *et al*., 2002), beta-

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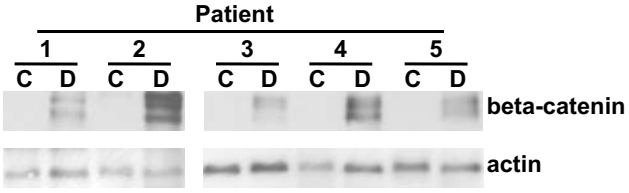

Figure 1 Western analysis of beta-catenin in DD. Western blot analysis was carried out using patient-matched disease and adjacent normal fascia (control) from five Dupuytren's patients. Protein extracts were prepared using a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 1 μg/ml of aprotinin, leupeptin, and pepstatin, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF). After centrifuging the extract to remove insoluble material, equivalent protein levels were subjected to Western analysis using an anti-beta-catenin monoclonal antibody (clone 14, Transduction Laboratories, Lexington, KY, USA), and an anti-actin antibody (Sigma, St Louis, MO, USA) to check for equal cellular protein loading. As shown here, beta-catenin is readily detectable within the disease (D) tissue, while little or no protein is detected within patient-matched normal/control (C) fascia

catenin (exon 3) does not appear to be mutated. This important regulatory region of beta-catenin contains several GSK-3-beta phosphorylation sites that are essential for the targeted degradation of beta-catenin via the ubiquitin-proteosome pathway (Hart *et al*., 1999; Latres *et al*., 1999; Winston *et al*., 1999). Mutations in this region of beta-catenin, which is commonly found in a number of human cancers (Polakis, 2000), result in protein stabilization leading to its accumulation within the nucleus where it interacts with members of the Tcf/Lef (T-cell factor-lymphoid enhancer factor) transcription factor family to activate gene expression (Korinek *et al*., 1997).

In light of the limited amount of information about the possible role of beta-catenin in DD, we have decided to further explore this protein–disease relations by using more extensive cell and biochemical approaches and both *in vivo* (clinical lesions) and *in vitro* (primary cell cultures) material. Initial Western analysis of several patient-matched DD specimens showed that lesional tissues expressed increasing amounts of beta-catenin compared to normal-appearing (control) fascia (Figure 1). Unlike diseased tissue, only very low to nondetectable levels of beta-catenin were seen in patient-matched control tissue specimens. IHC analysis of beta-catenin was also performed on formalin-fixed paraffin-embedded specimens, with the percentage of beta-catenin-positive staining and nuclear beta-catenin staining being assessed for cells within lesion and normal-appearing marginal tissue. Our analysis showed that the vast majority of cells within lesions expressed beta-catenin ($89 \pm 22\%$, $n = 12$ patients, 281 ± 58 cells/field, five fields/slide, one slide per patient), while cells within marginal tissue stained weakly for beta-catenin ($11.6 \pm 8.2\%$, $n = 12$ patients, 140 ± 95 cells/field, five fields/slide, one slide per patient). The overall lesional staining pattern for beta-catenin ranged from multiple intense focal regions to strong lesion-wide staining (Figure 2). Closer examination of beta-catenin-positive cells within the lesions showed it to be predominately cytoplasmic in nature, with varying degrees of positive nuclear staining ($42 \pm 24\%$).

Since elevations in beta-catenin levels are often the result of somatic mutations within beta-catenin itself, we also examined the mutational status of beta-catenin. To date, we have found no somatic mutations within exon 3 of beta-catenin (data not shown). These data are consistent with Montgomery *et al*. (2001) findings, which that show no mutations within either beta-catenin (exon 3), or the mutation cluster region adenomatous polyposis coli (APC) gene (Montgomery *et al*., 2001).

The tyrosine phosphorylation status of beta-catenin was also examined in DD. Western analysis of beta-catenin immunoprecipitated (IP) from several DD lesions and pooled control fascia tissue ($n = 5$ patients) showed that protein tyrosine phosphorylation is only detectable within lesional material (Figure 3). However, the very low levels of beta-catenin present within the control tissue may have prevented detection of any tyrosine-phosphorylated forms of the protein. The presence of smaller molecular weight bands recognized by both antiphosphotyrosine and anti-beta-catenin antibodies likely represent protease-cleaved forms of the protein, since the protein is actively targeted for degradation by the proteosome.

Finally, we examined beta-catenin levels *in vitro* using primary cell cultures established from patient-matched disease and normal (control) fascia. Monolayer cultures (passages 3–6, at either 50 or 100% confluency) of disease and normal primary cell lines showed similar levels of expression of beta-catenin and fibronectin, a known gene target of the beta-catenin/lymphoid enhancer factor (LEF) transcriptional complex (Gradl *et al*., 1999). However, when these cells were placed in three-dimensional collagen lattices to more closely replicate *in vivo* disease conditions (i.e. collagen-rich disease cords or nodules), significantly higher beta-catenin levels (normalized to Hsp47 levels) were observed in the disease lattices when compared to control lattices (3.7-fold, $P < 0.04$, Figure 4b). Beta-catenin levels in the control cells (normalized to Hsp47 levels) decreased significantly (2.5-fold, $P < 0.04$) upon transfer of these cells from monolayer cultures to three-dimensional collagen lattices. For disease cells, this same change in culture environments resulted in only a negligible decease in beta-catenin levels (1.03-fold). In addition, fibronectin levels in the disease cell lattices increased 1.8-fold when compared to control lattices, but these levels did not reach significance ($P = 0.069$). Similarly, for the disease cells the change in culture environments (monolayer to three-dimensional collagen lattice) resulted in a twofold increase in fibronectin levels ($P = 0.071$).

While the functional significance of beta-catenin in DD and the factors that regulate its expression are currently unknown, our results suggest that the high levels of beta-catenin are likely not because of mutations within beta-catenin (exon 3) itself. This is in agreement with an earlier IHC study of DD, which showed no mutations within either beta-catenin (exon 3) or the mutation cluster region of APC gene (Montgomery

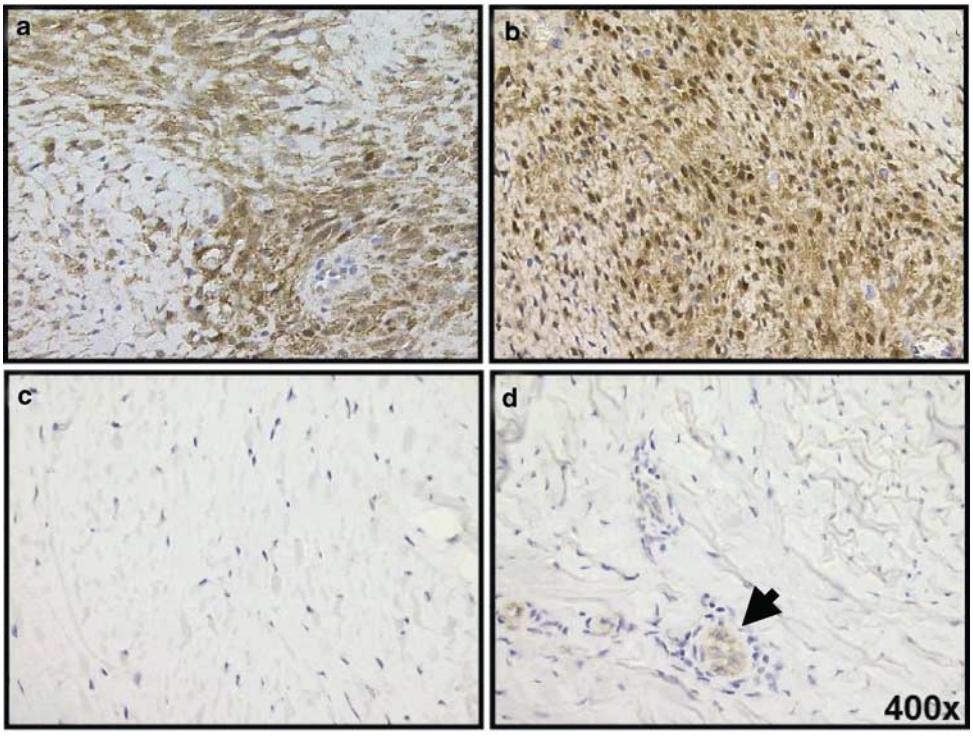

Figure 2 Beta-catenin immunohistochemistry of DD. Paraffin-embedded specimens were sectioned (4 μm), dewaxed, hydrated and treated with a 3% hydrogen peroxide solution to quench endogenous peroxidase activity. Antigen retrieval was aided by placing the slides in a preheated 10 mM citrate buffer (20 min). Slides were then rinsed in PBS, blocked with 10% normal horse serum (5 min), and then incubated with an anti-beta-catenin monoclonal antibody (1 h, 22°C, 1 : 50 dilution, clone 14, Transduction Laboratories). After a brief wash in PBS, the slides were incubated (30 min) with a biotinylated secondary anti-mouse antibody (Vector labs), washed briefly in PBS, and incubated (30 min, 22°C) with a tertiary antibody (Vector elite PK-6100). Finally, the slides were washed with PBS, and incubated (5 min) in di-amino benzidine (DAB). Sections were counterstained with hemotoxylin (1 min, Gills 3), dehydrated, cleared, and mounted with Micromount. (**a**, **b**) These sections ($\times$ 400) show intense beta-catenin staining throughout lesional tissue, with noticeable cytoplasmic and nuclear accumulations. (**c**, **d**) Sections ($\times$ 400) of marginal fascia tissue (control) shows some beta-catenin staining. The arrow points to more noticeable beta-catenin staining within endothelial cells

et al., 2001). Interestingly, we have documented a similar type of alteration in beta-catenin expression in a subset of Desmoid tumors (Tejpar *et al*., 1999), where 11 of the 42 tumors examined had high levels of beta-catenin expression, but no somatic mutations within either beta-catenin (exon 3) or APC (mutational cluster region).

Despite the absence of beta-catenin mutations, there are a number of possible mechanisms that may explain the high levels of beta-catenin seen in DD. These would include the secretion of wingless (Wnt) factors (Shimizu *et al*., 1997), alterations in other Wnt signal pathway components (Morin, 1999), or perhaps the activation of Wnt-independent signaling pathways that modulate beta-catenin stability. The latter possibility is an intriguing one since factors that could possibly fulfill this role might include transforming growth factor-beta (TGF-beta) isoforms. These fibrogenic cytokines have long been implicated in DD (Kloen, 1999), and are known to cooperate with Wnt proteins in regulating certain developmental events (Cadigan and Nusse, 1997). Recent studies have also shown that TGF-beta1 can activate beta-catenin expression in immortalized epithelial cells (Wang and Chakrabarty, 2001), while primary DD fibroblasts appear to secrete actively latent TGF-beta2 when cultured in three-dimensional collagen lattices (Kuhn *et al*., 2002). Regardless, it will be interesting to determine if TGF-beta isoforms can significantly alter beta-catenin levels in primary DD cultures, and, if so, what function it might serve in these disease cells.

The changes in beta-catenin expression observed in collagen lattice cultures raises the intriguing possibility that the extracellular matrix (ECM) may play an important role in modulating beta-catenin levels. The relative decrease in beta-catenin levels in control cells upon transfer from monolayers to three-dimensional collagen lattices appears to account, in large part, for the differences in beta-catenin levels between disease and control lattices. The ability of collagen to alter beta-catenin expression is perhaps not too surprising given the profound effect that adhesion to the ECM can have on cell behavior and tissue homeostasis (Boudreau and Bissell, 1998; Schwartz and Ginsberg, 2002). The potential role of collagen in modulating beta-catenin levels is also supported by studies of the antiangiogenic activity of endostatin. This C-terminal fragment of collagen type XVIII has been shown to block the transcriptional activity of beta-catenin, suggesting that ECM molecules may play an important role in modulating Wnt/beta-catenin signaling (Hanai *et al*.,

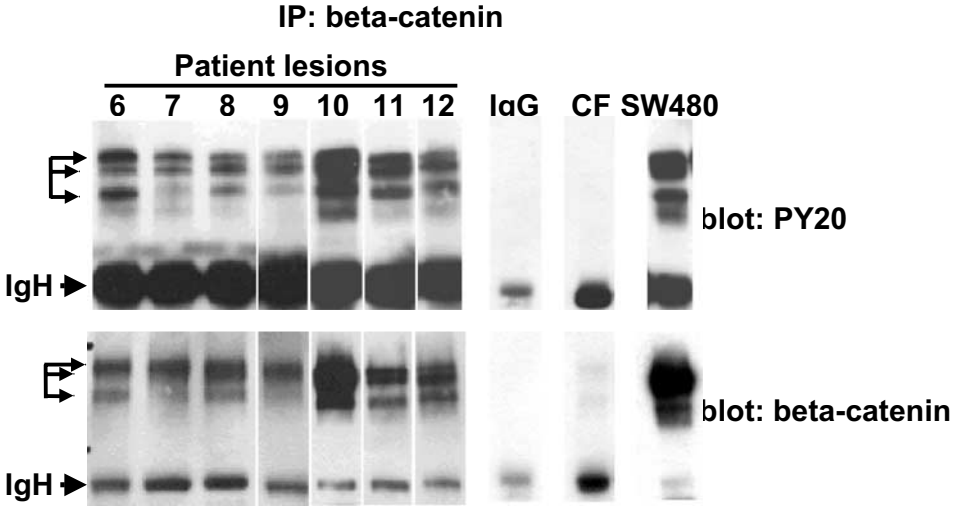

Figure 3 Western analysis of beta-catenin immunoprecipitates. Beta-catenin was IP from lesional tissue ($n=7$ patients) and pooled control fascia (CF, $n=5$ patients) using an anti-beta-catenin monoclonal antibody (clone 14, Transduction Laboratories). Protein extracts were prepared as in Figure 1. Samples (250 μg of protein) were precleared with protein-A agarose and then incubated with 5 μg of anti-beta-catenin antibody (2 h, 4°C). Protein–antibody complexes were captured with protein-A agarose (1 h, 4°C) and centrifuged. Pellets were washed with extraction buffer and resuspended in SDS sample buffer for Western analysis using the anti-phosphotyrosine antibody PY20 (Transduction Laboratories). As shown here, beta-catenin tyrosine phosphorylation is detected within lesional tissue (patients 6–12) and the colon carcinoma cell line SW480 (positive control), but not in the control fascia tissue. Nonimmune IgG was used as a negative IP control

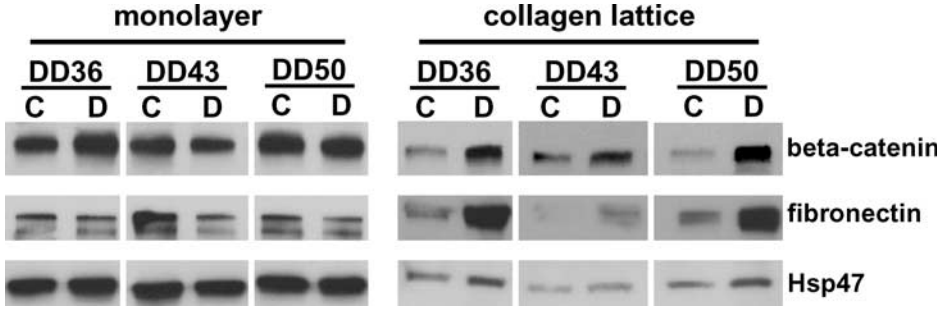

Figure 4 Western analysis of beta-catenin expression *in vitro*. Western analysis of beta-catenin was carried out using primary cell cultures established from three patient-matched diseases (D) and normal (C, control) fascia samples (DD36, 43, 50). Primary cell cultures were either grown as two-dimensional monolayers (plastic dishes), or as three-dimensional collagen lattices. Cell populated collagen lattices were prepared by mixing cells with a neutralized solution of Vitrogen100 collagen type I (eight parts Vitrogen100, 2.9 mg/ml, (Collagen Corp, Santa Clara, CA, USA) + one part 10 × α-MEM + one part HEPES buffer, pH 9). The cell–collagen mixture was dispensed into 24-well culture dishes (0.5 ml/well, $\sim 1.5 \times 10^5$ cells/well). Once polymerized (1 h, 37°C), culture media (α-MEM + 10% FCS) was added to each well. After 2 days of incubation, the lattices were harvested and homogenized using a modified RIPA buffer (see Figure 1 legend). Western analysis using anti-beta-catenin (clone 14, Transduction Laboratories) and anti-fibronectin (clone IST-4, Sigma, St Louis, MO, USA) antibodies detected elevated levels of beta-catenin and fibronectin in the disease lattices, while monolayer cultures showed similar levels of expression of both proteins in the disease (D) and control (C) cell cultures

2002a, b). Future studies of DD will hopefully explore the potential role of ECM molecules and growth factors in modulating beta-catenin stability and function.

Unlike Desmoid tumors, beta-catenin is tyrosine phosphorylated in DD. Although the functional significance of this tyrosine phosphorylation is unclear, a number of studies have shown a correlation between increased tyrosine phosphorylation of beta-catenin in response to v-src transformation or growth factor stimulation, and increased cell migration and invasion (Behrens *et al*., 1993; Shibamoto *et al*., 1994; Shibata *et al*., 1996; Muller *et al*., 1999). Recently we have shown, using mouse primary dermal fibroblasts carrying an inducible mutant beta-catenin transgene, significant increases in cell proliferation, motility, and invasiveness upon induction of the mutant transgene (Cheon *et al*., 2002). While the tyrosine phosphorylation levels of beta-catenin was not determined, the ability of high levels of beta-catenin to enhance normal fibroblast motility and invasiveness may help to explain some of the clinical aspects of DD, such as disease cord (collagen) contraction. This hallmark of DD involves extensive cell–cell interactions and cell–ECM remodeling events, activities regulated by key signaling molecules, including beta-catenin, which help coordinate events (cell adhesion, cytoskeletal reorganization, and the transmission of

mechanical forces) important to cell migration and contraction.

The current lack of knowledge about the underlying pathomechanisms of DD, coupled with the limited therapeutic intervention available (surgical resection of disease cords followed by extensive postoperative rehabilitation), emphasizes the need for a better understanding of the basic biology of DD. The elevated levels of beta-catenin detected in DD suggests that beta-catenin and/or the factors that regulate its stability may serve as potential therapeutic targets in the treatment of DD and perhaps other related fibroproliferative disorders, such as Peyronie's disease, aggressive fibromatosis (desmoid tumors), fibrosarcomas, and hypertrophic scars. Future studies are needed to explore the possible relationships between beta-catenin, ECM and growth factor receptor signaling in DD.

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