WT1 expression is increased in primary fibroblasts derived from Dupuytren’s disease tissues

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Abstract Dupuytren’s disease (DD) is a fibroproliferative and contractile fibrosis of the palmar fascia that, like all other heritable fibroses, is currently incurable. While DD is invariably benign, it exhibits some molecular similarities to malignant tumours, including increased levels of β-catenin, onco-fetal fibronectin, periostin and insulin-like growth factor (IGF)-II. To gain additional insights into the pathogenesis of DD, we have assessed the expression of WT1, encoding Wilm’s tumour 1, an established tumour biomarker that is syntenic with IGF2, the gene encoding IGF-II in humans. We found that WT1 expression is robustly and consistently up regulated in primary fibroblasts derived from the fibrotic palmar fascia of patients with DD (DD cells), whereas syngeneic fibroblasts derived from the macroscopically unaffected palmar fascia in these patients and allogeneic fibroblasts derived from normal palmar fascia exhibited very low or undetectable WT1 transcript levels. WT1 immunoreactivity was evident in a subset of cells in the fibrotic palmar fascia of patients with DD, but not in macroscopically unaffected palmar fascia. These findings identify WT1 expression as a novel biomarker of fibrotic palmar fascia and are consistent with the hypothesis that the pathogeneses of DD and malignant tumours have molecular similarities.

Keywords Dupuytren’s disease · Fibrosis · Wilm’s tumor 1 · Biomarker

Introduction

Palmar fibromatosis is often referred to as Dupuytren’s disease (DD) in deference to the French surgeon who was amongst the first to describe and treat this condition (Dupuytren 1834). It is a benign and heritable (Capstick et al. 2013) fibrosis that is initially evident as a nodule of myofibroblasts (Berndt et al. 1994; Bisson et al. 2003; Iwasaki et al. 1984; Magro et al. 1997; Tomasek et al. 1986) within the palmar fascia (palmar aponeurosis), a thin layer of connective tissue below the dermis in the palm. Over time, and through a poorly understood process, nodular myofibroblasts spread along the palmar fascia, secrete collagens and other extra-cellular matrix (ECM) proteins, and exert contractile forces on this collagen-enriched matrix. Contraction of these collagenous “cords” (Chiu and McFarlane 1978; Rayan 1999) result in the permanent finger contractures that characterize DD (Badalamente et al. 1996; Berndt et al. 1994; Magro et al. 1997; Tomasek et al. 1986; Tomasek et al. 1987). Depending on whether finger
contractures or the presence of palmar nodules are assessed as evidence of disease, the prevalence of DD is estimated to be between 1 and 7 % in the United States of America (Dibenedetti et al. 2012) and to be as high as 32 % in some regions of Europe (Degreel and De Smet 2012). As all of the available treatments for this fibrosis are associated with disease recurrence rates of 30 % or greater (Bulstrode et al. 2005; Foucher et al. 2003; Kan et al. 2015; Watt et al. 2012), DD is currently considered incurable.

Despite being characterized as benign, DD tissues and the primary fibroblasts derived from these tissues (DD cells) display some of the molecular characteristics of sarcomas and tumor stroma. These include, but are not limited to, increased β-catenin levels (Howard et al. 2004; Howard et al. 2003; Varallo et al. 2003), increased expression of FN type III extra-domain B (ED-B) and “oncofetal” fibronectin levels, increased POSTN expression and peristin levels (Vi et al. 2009) and increased IGF2 expression and insulin-like growth factor-II (IGF-II) levels (Raykha et al. 2013). We have interpreted these findings to suggest that the pathogenesis of DD and tumor/stroma development may involve the activation of similar molecular pathways (Bowley et al. 2007), and that fibrosis development may represent either an alternative outcome to, or a precursor of, tumor development.

Increased IGF2 expression and/or increased β-catenin levels are common features of many different cancers (Alman et al. 1997; Barker and Clevers 2000; Cui 2007; de Groot et al. 2007; Heaton et al. 2013; Lu et al. 2006; Merle and Trepo 2009; Morin 1999; Shah et al. 2002; Singh et al. 1998; Tetsu and McCormick 1999) including Wilm’s tumors (Fukuzawa et al. 2008; Haruta et al. 2008; Md Zin et al. 2013), a type of paediatric kidney tumour. Wilm’s tumors are best known for featuring inactivating mutations of WT1, encoding the alternatively spliced zinc finger transcription factor (Caricasole et al. 1996; Magro et al. 2014) and RNA splice factor (Caricasole et al. 1996; Hewitt and Saunders 1996; Kennedy et al. 1996) Wilm’s tumor 1 (WT1). Despite its original identification as a tumour suppressor gene (TSG) in Wilm’s tumors, WT1 expression is frequently up regulated in other tumours where it is considered to be both oncogenic and a biomarker of tumour sub-type (Nakatsuwa et al. 2006; Ohno et al. 2009; Sebire et al. 2005; Shimizu et al. 2000; Wilsher and Chee rala 2007). As WT1 and IGF2 are syntenic on chromosome 11p and some of their transcripts are subject to alterations in genomic imprinting in tumours (Haruta et al. 2008; Jacobs et al. 2013; Malik et al. 2000; Mitsuya et al. 1997), we were curious to see if WT1 expression, like IGF2 expression (Raykha et al. 2013), was dysregulated in DD. Here we report that WT1 expression is robustly and consistently increased in DD cells relative to both syngeneic fibroblasts derived from the visibly non-fibrotic palmar fascia and allogeneic fibroblasts derived from normal palmar fascia. WT1 immunoreactivity was evident in discrete subsets of cells within fibrotic palmar fascia, but not in macroscopically unaffected palmar fascia. These findings implicate WT1 as a novel biomarker of this fibrosis and support our hypothesis that the pathogenesis of DD and tumour development share overlapping molecular characteristics.

Methods

Derivation of primary fibroblasts

Palmar fascia tissue samples were resected from patients with Dupuytren’s disease (DD) and from patients undergoing carpal tunnel release (CT) during surgeries at the Roth McFarlane Hand and Upper Limb clinic. All patients received a letter of information and signed consent forms for their tissues to be used for research purposes and the samples were collected with the approval from the University of Western Ontario Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB protocol # 104888). Patient de-identification and confidentiality were achieved by assigning lab numbers to the samples prior to processing. Primary DD fibroblasts were derived from visibly fibrotic palmar fascia (DD cells), and from phenotypically unaffected adjacent palmar fascia of the same patient (PF cells), as syngeneic controls. Normal palmar fascia fibroblasts were derived from patients with no prior history of Dupuytren’s Disease undergoing carpal tunnel release (CT cells) as allogeneic controls.

Real-time quantitative PCR analyses

Total RNA samples from primary DD, PF and CT cells were assessed for quality on a NanoDrop spectrophotometer ND-1000. 2 μg of high quality total RNA was reverse transcribed into cDNA first strand using the High-Capacity cDNA Archive Kit (Applied Biosystems) in accordance with the manufacturer’s instructions. TaqMan gene expression assays were used to measure WT1 mRNA levels (Hs01103751_m1) relative to the RPLP0 endogenous control (Hs99999902_m1) using the ΔΔCt method after confirmation of parallel PCR amplification efficiencies on a Real-Time PCR ABI Prism 7500. PCR reactions were carried out under the following conditions: Initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation (95 °C for 15 s), primer annealing (60 °C for 1 min) and transcript extension (50 °C for 2 min).

Immunohistochemistry

Surgically resected fibrotic (N=2) and macroscopically non-fibrotic (N=2) palmar fascia samples were resected from patients undergoing fasciectomies...
The tissues were fixed in 10 % formalin, dehydrated, paraffin embedding and sectioned. Sections (5 μm) were dewaxed, rehydrated, and processed for antigen retrieval using standard citrate-based protocols. Sections were rinsed in PBS prior to incubation with WT1 polyclonal antibody (clone 6F-H2 in "ready to use" dilution buffer, Dako Cat# IR055). Sections were counterstained with Gills hematoxylin for 1 min and visualized by light microscopy.

Results

**WT1 expression in DD, PF and CT cells** WT1 expression was assessed in syngeneic DD and PF cells from 13 patients and in allogeneic CT cells from 6 patients. As shown in Fig. 1, a mean increase in WT1 expression of ≥25 fold was evident in DD cells relative to both PF cells and CT cells cultured under identical conditions in αMEM supplemented with 10 % FBS. WT1 transcripts could not be detected in approximately 50 % (7/13) of the PF cell cultures and in 17 % (1/6) of the CT cell cultures within the 40 PCR cycle limit. In the remainder of the PF and CT cell cultures, WT1 transcripts were detected at an average of 35 and 37 PCR cycles respectively, indicative of very low levels of WT1 mRNA transcripts in these cells. In contrast, WT1 expression was detected in 100 % of the DD cell cultures assessed at an average of 28 PCR cycles, and WT1 transcripts were invariably (13/13) detected at a lower cycle of PCR cycles in the DD cells than in the PF cells derived from the same patient.

**WT1 immunoreactivity in surgically resected DD tissues** Paraffin-embedded fibrotic and macroscopically unaffected palmar fascia tissues were sectioned and assessed for
WT1 immunoreactivity as described in the methods. As shown in Fig. 2a, b and c, discrete clusters of WT1-positive cells were observed throughout the sections of fibrotic palmar fascia. In contrast, no WT1 immunoreactivity was observed in any of the macroscopically unaffected palmar fascia tissue sections assessed (Fig. 2d).

Discussion

To our knowledge, this is the first report of increased WT1 expression and WT1 immunoreactivity in DD. The WT1-immunoreactive cells identified in DD tissues made up approximately 10% of the total number of cells in these tissues and were typically clustered together, implying that they may represent a distinct sub-population of cells that are specific to fibrotic, but not normal, palmar fascia. The relative scarcity of WT1 positive cells in DD tissues contrasted to the consistent, high-level expression of WT1 in primary cells derived from these tissues. It is currently unclear if WT1-positive cells are preferentially isolated during explant cultures or whether some aspect of in vitro culture enhances WT1 expression. WT1 gene transcripts are subject to extensive alternative splicing in other disease systems (Bickmore et al. 1992; Hewitt and Saunders 1996; Morrison et al. 2008) and the translated products of these variants may include or exclude a region encoding a Lys-Thr-Ser (KTS) tripeptide, resulting in WT1 + KTS and WT1-KTS protein isoforms (Charløe et al. 1995; Lee and Haber 2001; Lee et al. 1999; Morrison et al. 2006). The WT1-KTS isoform has been reported to localize to the nucleus, bind DNA and function as a zinc-finger transcription factor to activate or repress gene transcription, whereas the WT1 + KTS isoform is proposed to interact with factors that regulate RNA splicing in the cytoplasm (Morrison et al. 2006). The nuclear or cytoplasmic localization of WT1 varies between tumor types and is used for tumor characterization (Hecht et al. 2002; Magro et al. 2014; Nakatsuka et al. 2006; Sebire et al. 2005), however it is unclear if the cellular location of WT1 immunoreactivity strictly correlates with the relative abundance of + KTS and -KTS isoforms. While WT1 immunoreactivity appeared to be mostly localized to the cytoplasm of cells in fibrotic palmar fascia tissues, further analyses will be required to confirm this observation and any correlation with WT1 isoform expression.

IGF2 and WT1 are syntenic on the short arm of chromosome 11, at 11p15.5 and 11p13 respectively, and each express a subset of transcripts that are subject to genomic imprinting, or parent-of-origin-specific gene expression, in various tissues. Loss of IGF2 and WT1 imprinting in tumors typically results in up regulated expression levels (Brown et al. 2008; Jacobs et al. 2013), and our recent unpublished findings suggest that IGF2 imprinting may be lost in a subset of patients with DD. As both IGF2 and WT1 expression levels are up regulated in DD cells, we will include WT1 in these ongoing studies to determine if abnormal epigenetic regulation of expression contributes to the increased IGF2 and WT1 transcript levels in this fibrosis.

Depletion of WT1 levels in idiopathic pulmonary fibrosis has been reported to inhibit myofibroblast formation (Karki et al. 2015) and increased WT1 expression in DD myofibroblasts may imply a similar role for WT1 in their development. If WT1 depletion in DD is found to inhibit myofibroblast development, it may be feasible to cross-purpose the WT1 peptide-based immuno-therapies currently under clinical investigation as treatments for a variety of cancers (Dohi et al. 2011; Dubrovsky et al. 2014; Elmaagacli et al. 2005; Nishida et al. 2014; Oka et al. 2002; Oka et al. 2006; Shirakata et al. 2012) as anti-fibrotic interventions. While the efficacy of this novel approach is yet to be clearly demonstrated, the potential to attenuate fibrosis-associated myofibroblast development by immunizing patients with WT1 peptides is intriguing and may be worthy of further investigation.

References


