Biomarkers of Postsurgical Outcome in Dupuytren Disease

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7.1 Introduction

Dupuytren Disease (DD) is a common disabling condition of the hand, affecting over 2 million people in the UK (Shaw et al. 2007; Townley et al. 2006). It is characterised by fibrosis of the palmar fascia. Initially there is a cellular hyperproliferation to produce nodules with deposition of collagen-rich cords. The combination of a cell-mediated contraction and remodelling of the extracellular matrix (ECM) leads to shortening of the tissue and deformity of the fingers (contracture). The only current treatment for Dupuytren contracture is surgery with high recurrence rates or minimally invasive treatments, like collagenase injection or needle fasciotomy, with even faster recurrence. Further surgery becomes more invasive with higher complication rates and incomplete contracture correction. There are no known drug treatments for the condition at present. The cellular and molecular mechanisms leading to Dupuytren contracture are poorly understood.

The matrix metalloproteinases (MMPs) are a family of 23 enzymes in man, including enzymes able specifically to degrade collagen (MMP-1, MMP-2, MMP-8, MMP-13 and MMP-14) (Kessenbrock et al. 2010). A related family of 19 metalloproteinases, the ADAMTSs, include the major aggrecan-degrading proteinases and three procollagen N-propeptidases which are

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important in the synthesis of collagen (ADAMTS-2, ADAMTS-3 and ADAMTS-14) (Kelwick et al. 2015). There are four specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), with varying selectivity against the proteases (Brew and Nagase 2010). Normal ECM turnover requires a balance between metalloproteinase and inhibitor activities with fibrosis coming from an imbalance away from proteolysis (Kessenbrock et al. 2010). The MMPs are implicated in tumour invasion and metastasis. When MMP inhibitors underwent clinical trials in cancers (Vandenbroucke and Libert 2014), the major side effect was a 'musculoskeletal syndrome', described as frozen shoulder and/or a condition resembling DD (Hutchinson et al. 1998). The inhibitors were 'pan-MMP' inhibitors, showing ~ nanomolar Ki against many of the MMPs tested. The musculoskeletal syndrome is ascribed to the inhibition of non-target metalloproteinases both within and outside of the MMPs.

Whilst subsets of MMPs and TIMPs had been measured in DD tissue, we assayed the expression of the entire *MMP*, *TIMP* and *ADAMTS* gene families in DD tissue (nodule and cord) compared to normal palmar fascia using qRT-PCR. The expression of four key collagen-degrading proteinases, *MMP1*, *MMP2*, *MMP13* and *MMP14* as well as *TIMP1*, was significantly raised in DD nodule, as was the expression of a collagen biosynthetic enzyme *ADAMTS14*. We concluded that palmar fibrosis and finger contracture in DD may result from (i) increased collagen biosynthesis with processing mediated by increased ADAMTS-14, (ii) elevated TIMP-1 blocking MMP-1- and MMP-13-mediated collagenolysis (with these enzymes elevated in an attempt to resolve the fibrosis), and (iii) contraction enabled by MMP-14 (and MMP-2-)-mediated pericellular collagenolysis which escapes inhibition by TIMP-1 (Johnston et al. 2007). We also followed DD patients for 2 years and assessed their hand function and disease recurrence after surgery. We discovered that the expression of key MMPs (e.g. *MMP1*, *MMP2*, *MMP13* and *MMP14*) and *TIMP1* correlates with poor progression post-fasciectomy (Johnston et al. 2008). This reinforces their role as key mediators of the disease process.

Recently we have used *in vitro* models of cell-mediated contraction to investigate the function of key proteinases and inhibitors that we have identified in DD tissue (Wilkinson et al. 2012). In fixed fibroblast-populated collagen lattice (FPCL) models, broad spectrum synthetic MMP inhibitors block contraction (Daniels et al. 2003; Townley et al. 2008). This blockade is difficult to dovetail with the reported side effects of pan-MMP inhibitors in clinical trials, where Dupuytren-like contracture was observed (Hutchinson et al. 1998). However, this reflects our increasing understanding that MMPs can mediate positive, as well as negative, effects (Kessenbrock et al. 2010), and the need is to identify specific MMPs as therapeutic targets (Vandenbroucke and Libert 2014). Dupuytren fibroblasts do show increased contraction in the FPCL model, which is a relevant feature of DD (Bisson et al. 2004). It was therefore important both to understand the role of specific metalloproteinases in the model. We profiled expression of all *MMPs*, *ADAMTSs* and *TIMPs* in DD cells within FPCL both during the development of tension and following release (Wilkinson et al. 2012). *MMP1*, *MMP2*, *MMP3*, *MMP13*, *MMP14* and *TIMP1* all show increased expression in the 3D collagen lattice compared to monolayer culture. *MMP1*, *MMP3*, *MMP14* and *TIMP1* all then show decreased expression under tension, with increase upon release, whilst *MMP13* shows a steady increase across the assay that may simply reflect response to the collagen lattice, and *MMP2* is not regulated at this level. To examine the role of each of MMP-1, MMP-2, MMP-3, MMP-13 and MMP-14, an siRNA approach was developed to knock each gene down individually. Knockdown of *MMP2* and particularly *MMP14* led to decreased contraction of the collagen lattice with slower kinetics, demonstrating a key role for these proteinases. Knockdown of *MMP1* gave a more rapid contraction in the early phase of the assay, leading us to speculate that the action of MMP-1 may be

to decrease tension in the fixed phase of the lattice. Knockdown of *MMP3* or *MMP13* had no significant effect on contraction, and therefore these proteases do not have an essential role in contraction.

Leading on from the correlation between gene expression and tissue levels of specific MMPs and their role in contraction, we hypothesised that circulating levels of collagen-degrading MMPs, MMP-1, MMP-13 and MMP-14 may be biomarkers of disease progression and/or postsurgical recurrence. If proven, this could lead to a blood test which would guide surgical decision making. MMPs (MMP-1, MMP-2, MMP-9) and TIMPs (TIMP-1 and TIMP-2) have been measured in the sera of patients with DD compared to patients undergoing carpal tunnel release, with significantly higher TIMP-1 levels measured in DD patients (Ulrich et al. 2003). However, these data were not compared to measurements of DD.

7.2 Materials and Methods

7.2.1 Patient Samples

All surgery was performed at the Norfolk and Norwich University Hospital under approval from the local research ethics committee; all patients gave informed consent. Dupuytren Disease tissue was taken at fasciectomy ($n=25$, age range 50–78 years, 5 female, 20 male). Samples were divided into regions of nodule and cord according to gross morphology. Normal palmar fascia was taken from patients without DD undergoing carpal tunnel release ($n=30$, age range 31–88 years, 20 female, 10 male). Tissue was dissected into approximately 5 mm pieces and snap frozen in liquid nitrogen within 15–30 min of surgery. Blood was taken from patients immediately prior to surgery into sodium citrate. Within 60 min, it was centrifuged at 1600 g for 15 min at 4 °C and plasma removed and stored in aliquots at −80 °C. Total extension deficit was measured with a goniometer, with deficit from affected digits added together.

7.2.2 RNA Isolation and qRT-PCR

TRIzol reagent (Life Technologies) was used to isolate total RNA from tissue, with the aqueous fraction from the phase separation further purified using the RNeasy Mini Kit (Qiagen). Reverse transcription used 1 μg total RNA (DNase treated) and SuperScript III with random hexamers. Data from qRT-PCR was normalised to expression of 18S ribosomal RNA. Fluorescence for each cycle was analysed by the real-time PCR 7500 system (Applied Biosystems).

7.2.3 Microarray Analyses

Profiling of mRNA used the Illumina Human HT12v4 platform (Source Bioscience). Data were analysed using the Bioconductor packages (http://www.bioconductor.org) in R (http://www.r-project.org). Data were firstly preprocessed by background correction, variance stabilisation and normalisation using the quantile method.

7.2.4 Enzyme-Linked Immunosorbent Assays (ELISA)

Commercial kits were used to measure MMPs by ELISA according to manufacturer's instructions. MMP-1, R&D Systems DuoSet, human total MMP-1 #DY991; MMP-13, R&D Systems DuoSet, human total MMP-13 #DY511; MMP-14, Cloud Clone US #SEC056Hu. Samples were diluted 1:2 for measurement of both MMP-1 and MMP-14 and 1:4 for measurement of MMP-13.

7.2.5 Statistics

Correlation analyses used either Pearson correlation for normally distributed data or Spearman's rank correlation for non-parametric data. For array data, the rank of their expression level across all samples was computed and compared to the rank of clinical measurements to give the Spearman's rank correlation.

7.3 Results

7.3.1 Measurement of Total Extension Deficit (TED)

In patients undergoing fasciectomy ($n=25$), a number of measurements of hand function were taken preoperatively and then at an intermediate time (up to 12 weeks) after surgery and postoperatively after approximately 1 year. Total extension deficit (TED) is a measure of contracture of the digits. Figure 7.1 shows that 24 patients had the expected decrease in TED after surgery. Between the intermediate and postoperative measurements, 9 patients have continued decrease in TED, 4 patients have no change (all have zero TED) and 5 have increased TED, with 7 having incomplete data.

7.3.2 Correlation of Gene Expression with TED

Initially, the expression of *MMP1*, *MMP13* and *MMP14* was measured at the mRNA level in Dupuytren Disease tissue taken at fasciectomy. Gene expression was correlated with TED taken preoperatively, immediately postoperatively (intermediate) and then at one year postoperatively (as in Sect. 7.3.1). Compared to our previous study (Johnston et al. 2008), there was little correlation in this cohort, with a statistically significant correlation between MMP1 expression and the change between preoperative and one year postoperative TED (Fig. 7.2a). A correlation was also measured between MMP14 expression and the change between intermediate and one year postoperative TED, approaching statistical significance (Fig. 7.2b).

7.3.3 Correlation of Circulating MMPs with TED

The level of MMP-1, MMP-13 and MMP-14 was measured in plasma taken preoperatively and correlated with measurements of TED. Whilst there was no correlation with postoperative progression of TED (i.e. disease recurrence), a correlation was seen between the circulating level of MMP-14 and preoperative TED (Fig. 7.3) which approached statistical significance.

7.3.4 Correlation of Gene Expression with TED

RNA from ten Dupuytren nodules was subjected to whole genome array to measure total gene expression. These were then subjected to correlation

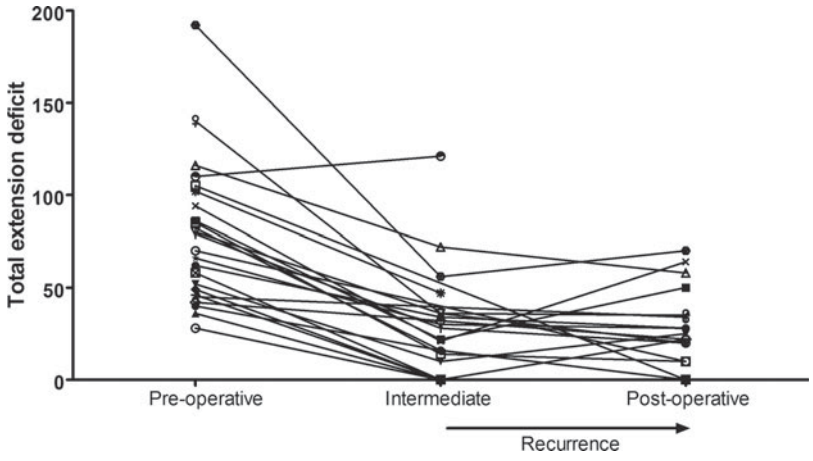

Fig. 7.1 Measurement of total extension deficit. Patients ($n=25$) underwent fasciectomy for Dupuytren Disease. Total extension deficit was measured with a goniometer, with deficit from affected digits added together. Measurement was taken preoperatively, at an intermediate stage (up to 12 weeks after surgery), and then postoperatively at approximately 1 year

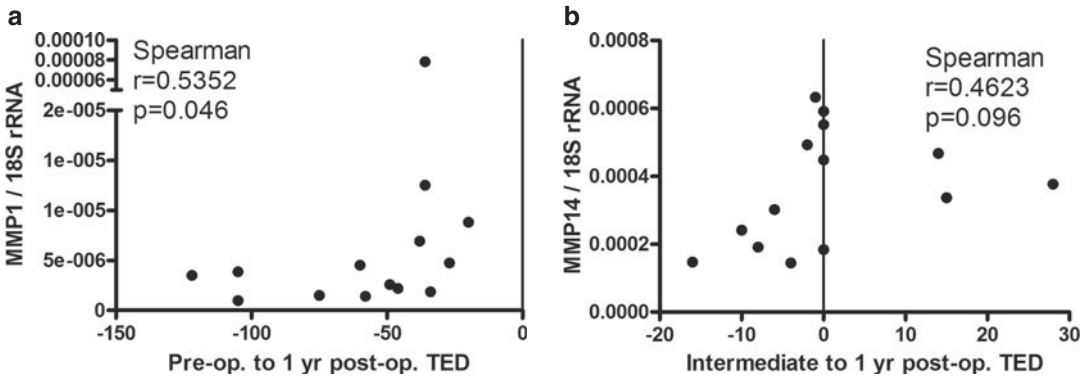

Fig. 7.2 Correlation between gene expression in nodule tissue and total extension deficit. Expression of matrix metalloproteinase (*MMP*) gene expression ((**a**) *MMP1* and (**b**) *MMP14*) was measured by qRT-PCR in tissue (nodule) taken at fasciectomy ($n=14$) and correlated with change in total extension deficit

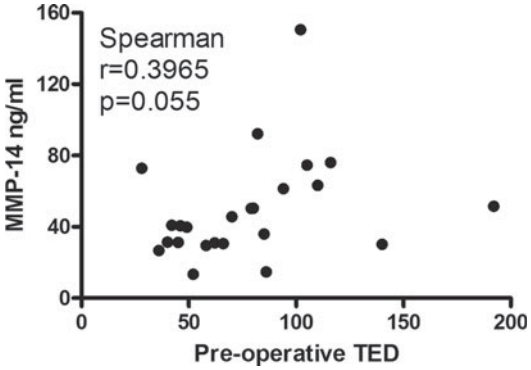

Fig. 7.3 Correlation between measurement of circulating MMP-14 and total extension deficit. Matrix metalloproteinase-14 (*MMP-14*) was measured in the plasma of patients with Dupuytren Disease ($n=25$) in advance of fasciectomy and correlated with total extension deficit

analyses. Interestingly, the expression of a number of genes was correlated with either preoperative TED (e.g. ASPN, asporin, Fig. 7.4a) or postoperative TED (e.g. fucosyltransferase 1, FUT1, Fig. 7.4b).

7.4 Discussion

There are a number of genes/proteins associated with DD, coming from genetic studies, e.g. Dolmans et al. (2011); microarray analyses, e.g. Shih et al. (2012b); or targeted approaches, e.g. Raykha et al. (2013). Any of these proteins which are extracellular have the potential to be biomarkers of disease.

We sought to explore the utility of collagen-degrading proteases (MMP-1, MMP-13 and MMP-14) as circulating biomarkers of postsurgical recurrence of Dupuytren Disease after fasciectomy. Twenty-five patients undergoing fasciectomy were recruited, taking a blood sample preoperatively. Tissue was then collected at surgery for gene expression analyses.

Measurement of TED showed the expected decrease immediately postoperatively, with patients then progressing either to a further decrease in TED or an increase. This increase in TED between the immediately postoperative stage and then at one year was deemed to be recurrence of disease.

Whilst our earlier studies had shown correlation between the tissue expression of *MMP1*, *MMP13* and *MMP14* and disease recurrence defined in this way, there was only poor correlation in the current study. This may simply reflect variation in patient cohorts.

Similarly there was no correlation between disease recurrence and the circulating level of any of the MMPs. However, there was a correlation between circulating MMP-14 and preoperative TED which adds to evidence from ourselves (Johnston et al. 2007, 2008; Wilkinson et al. 2012) and others (e.g. Shih et al. 2012a) that MMP-14 has a role in the mechanisms underlying Dupuytren Disease.

There are many reasons why gene expression and protein levels might not correlate and there are a number of issues in measurement of the

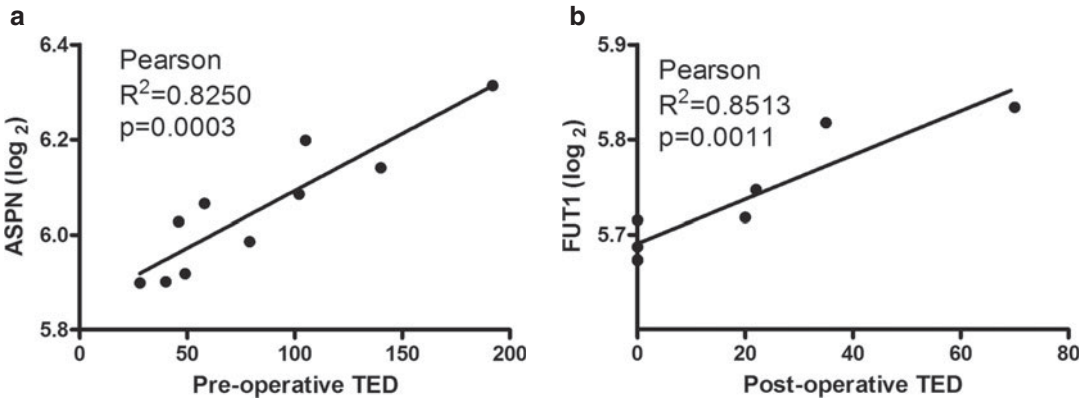

Fig. 7.4 Correlation between gene expression by microarray and total extension deficit. Gene expression was measured in a subset of Dupuytren patients ($n = 10$) by microarray. Gene expression was correlated with total extension deficit. (**a**) ASPN, asporin; (**b**) FUT1, fucosyltransferase 1

proteins. MMPs are synthesised as proenzymes, which are activated concomitant with the removal of their propeptide; the active enzymes can then be inhibited by TIMPs, giving an enzyme/inhibitor complex (Vandenbroucke and Libert 2014). ELISA assays, which rely on the recognition of specific epitopes by antibodies, are unlikely to measure all of these forms, and so there may not be a direct correlation between mRNA and protein measured by ELISA. Also, MMP-14 is a membrane-bound protease, so its level in the circulation is dictated by its shedding from the cell membrane (Itoh 2015). These all have the potential to confound the data. Furthermore, measurement of protein does not provide information about activity itself for these proteinases.

An unbiased approach, measuring the entire transcriptome, uncovered many genes whose expression correlates with either preoperative or postoperative TED (of which two are shown in Fig. 7.4). The measurement of these genes needs to be confirmed using qRT-PCR in order to demonstrate that they are not false-positives derived from multiple testing.

Conclusions

There is a strong need to identify biomarkers with which either to stratify Dupuytren Disease or to predict disease progression or recurrence. Whilst our data suggest that the collagen-degrading MMPs do not fit these criteria, the correlation of circulating MMP-14 with preoperative TED suggests its involvement in the disease process. Genes (and the proteins they encode) identified through high-throughput transcriptomics will be further pursued.

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