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TGF-β signalling-related markers in cancer patients with bone metastasis

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Abstract
We measured transforming growth factor (TGF)-β-dependent biomarkers in plasma and in peripheral blood mononuclear cells (PBMCs) to identify suitable pharmacodynamic markers for future clinical trials with TGF-β inhibitors. Forty-nine patients with bone metastasis were enrolled in the study, including patients with breast ($n=23$) and prostate cancer ($n=15$). Plasma TGF-β1 levels were elevated in more than half of the cancer patients (geometric mean 2.63 ng ml^{-1}) and positively correlated with increased platelet factor 4 (PF4) levels, parathyroid-related protein (PTHrP), von Willebrand Factor (vWF) and interleukin (IL)-10. PBMC were stimulated *ex vivo* to determine the individual biological variability of an *ex vivo* assay measuring pSMAD expression. This assay performed sufficiently well to allow its future use in a clinical trial of a TGF-β inhibitor.

Keywords: *TGF-β, plasma biomarkers, cancer*

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Introduction

Since its discovery in the early 1980s, transforming growth factor (TGF)-β was found to have tumour-inhibitory as well as tumour-promoting activity (Roberts et al. 1981, Sporn & Roberts 1985, Akhurst et al. 1988). TGF-β signalling affecting tumour growth can involve changes of the TGF-β receptor type-I (TGF-βRI), receptor type-II (TGF-βRII), and SMAD2 and SMAD3 signalling protein (Wakefield & Roberts 2002). The exact mechanisms leading to TGF-β-dependent tumour growth are still being investigated (Akhurst & Derynck 2001), and are important for the clinical development of TGF-β inhibitors (Yingling et al. 2004, Lahn et al. 2005). Various

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mechanisms have been proposed to explain the tumour-promoting ability of TGF-β (Dumont & Arteaga 2000, Akhurst & Derynck 2001). One of the mechanisms associated with TGF-β-mediated tumour growth is the enhancement of skeletal metastasis in breast and prostate cancer (Yin et al. 1999, Kang et al. 2001, 2005, Bandyopadhyay et al. 2006, Li et al. 2006). This process includes TGF-β-dependent activation of interleukin (IL)-11, the osteoclast-activating factor parathyroid-hormone-related peptide (PTHrP), vascular endothelial growth factor (VEGF), plasminogen activator inhibitor 1 (PAI-1) and urokinase plasminogen activator (uPA) (Siegel et al. 2003).

In the present study, we measured plasma TGF-β1 by enzyme-linked immunosorbent assay (ELISA) to determine its levels in patients with skeletal metastasis. Based on the observation of animal studies (Kang et al. 2005, Bandyopadhyay et al. 2006, Li et al. 2006), we compared plasma TGF-β1 levels with previously described TGF-β-associated circulating markers. We also determined the relationship between TGF-β1 levels and a panel of 79 different circulating markers of disease. Finally, we correlated the TGF-β1 levels with phosphorylation of SMAD (pSMAD) in PBMCs. We found that pSMAD levels in PBMCs are only detected after *ex vivo* stimulation. Our observations are useful in the application of these assays to measure pharmacological activity of TGF-β inhibitors in clinical trials.

Patients and methods

Patient selection

Between August 2004 and February 2005, 49 patients with skeletal metastasis were enrolled in a non-drug interventional study to donate blood. To be eligible in the participation of this study, patients had to fulfil the following criteria: signing informed consent in accordance with federal, state and institutional guidelines; be at least 18 years of age; have a previously documented diagnosis of histological or cytological advanced or metastatic cancer involving the bone; able to safely donate approximately 100 ml of blood over a 2–4-week period; and not receiving any chemotherapy or radiotherapy while in the study. Furthermore, if patients had been previously treated with chemotherapy or radiotherapy, the treatment had to be completed at least 14 days before enrolment. If patients had been previously treated with a drug that had not received regulatory approval for any indication, the last dose of such a drug had to be taken 30 days prior to study entry.

Patient visit and collection schedule

Patients had one venipuncture at each visit for a maximum of four visits. Visits were scheduled a minimum of 48 h and a maximum of 7 days apart over a 2–4-week period (Figure 1). At visit 1, blood samples were collected for TGF-β1, platelet factor 4 (PF4), β-thromboglobulin (βTG), PAI-1, VEGF, uPA, IL-11, PTHrP, multianalyte immunoassay panel (MAIP) and SMAD assay, including phosphorylated SMAD (pSMAD) and total SMAD (tSMAD). Patient information was also recorded, including medical history, concomitant medications and adverse events. At visits 2–4, concomitant medications and adverse events were documented. Also, plasma samples for TGF-β1, TG and PF4 were collected. At visits 2–4, PBMCs were

	Blood Draws at Each Visit			
Assays	**Visit 1**	**Visit 2**	**Visit 3**	**Visit 4**
TGF-β1, PF4, βTG ELISA	X	X	X	X
PAI-1, VEGF, uPA IL-11, PTHrP ELISA	X			
Multianalyte Immunoassay	X			
SMAD ELISA	X	X	X	X

Figure 1. Study design of patient visits and blood draws. At an interval of 2–7 days, patients were asked to donate blood for various assays. One sample was used to determine plasma levels of TGF-β1 under conditions of reduced thrombocyte activation (see Material and methods). Second and third samples were used to determine levels of TGF-β-dependent markers (PAI-1, VEGF, uPA) and a multianalyte immunoassay panel of 79 circulating proteins (Rules Based Medicine, Multianalyte Immunassay Panel, MAIP), respectively. A fourth sample was used to determine SMAD (pSMAD and tSMAD) expression in PBMCs after *ex vivo* stimulation with TGF-β1.

obtained for baseline and *ex vivo* assays to determine pSMAD and tSMAD expression.

Sample processing

TGF-β1, βTG and PF4 plasma levels. After the tourniquet was released, a single discard tube of approximately 10 ml blood was drawn to minimize potential effects of localized platelet activation caused by the tourniquet. Subsequently, and to further reduce preanalytical variation in TGF-β1 measurements caused by *ex vivo* platelet activation, blood was drawn into a Vacutainer tube containing citrate solution, theophylline, adenosine and dipyridamole (CTAD) (Becton Dickinson, Franklin Lakes, NJ, USA). The CTAD tube was processed as recommended by the manufacturer and plasma was stored at -70°C until analysis for TGF-β1, βTG and PF4 by Esoterix (Austin, TX, USA) using a validated ELISA method. The assay ranges for limit of quantification (LLQ) and higher/upper limit of quantification (HLQ) were provided, respectively, for TGF-β1 (0.75 ng ml^{-1}, 240 ng ml^{-1}), PF4 (3.4 IU ml^{-1}; no HLQ defined), and βTG (12.5 IU ml^{-1}, no HLQ defined) by Esoterix. Biological variability was defined based on a reference population using median, 5th percentile and 95th percentile, respectively, for TGF-β1 (1.344 ng ml^{-1}; 0.719–1.999 ng ml^{-1}), PF4 (12 IU ml^{-1}; 0–35 IU ml^{-1}), and βTG (49 IU ml^{-1}; 10–78 IU ml^{-1}). Assay variability was not provided by the vendor.

PAI-1, VEGF, uPA, IL-11, PTHrP. Seven millilitres of blood was collected into a glass vacutainer tube containing EDTA. Immediately following collection, the tube was gently rocked several times. Two millilitres of blood was transferred to a tube containing 1.2 trypsin inhibitor units of aprotinin (Sigma, St Louis, MO, USA). The tube was rocked several times and centrifuged for 15 min at 1600*g* at 4°C. The plasma was transferred to a fresh tube and stored at -70°C until analysis for PTHrP levels by Pathway Diagnostics (Malibu, CA, USA). The remainder of the EDTA sample was

centrifuged for 15 min at 2500*g* at 4°C. The plasma was transferred to a fresh tube and stored at −70°C until analysis of PAI-1, VEGF, uPA and IL-11 levels by Pathway Diagnostics using a validated ELISA method. The assay ranges for LLQ and HLQ, respectively, were provided for PTHrP (0 pg ml^{-1}; 22.3 pg ml^{-1}), PAI-1 (7.94 ng ml^{-1}; 59.61 ng ml^{-1}), uPA (504 pg ml^{-1}; 2595 pg ml^{-1}) and VEGF (0; 115 pg ml^{-1}). Assay or biological variability was not provided by the vendor.

Multianalyte Immunoassay Panel (MAIP). Seven millilitres of blood was collected into a glass vacutainer tube containing EDTA. The tube was gently mixed 8–10 times and centrifuged at room temperature for 15 min at 2500*g*. The plasma was transferred to a fresh tube and stored at −70°C until analysis by Rules Based Medicine (RBM, Austin, TX, USA) using a fluorocytometric analysis platform for the MAIP. Details on the MAIP are found at the website of RBM at www.rulesbasedmedicine.com.

SMAD assay (Figure 2). A detailed description on establishing the assay is provided elsewhere (Farrington et al. 2007). Briefly, blood was drawn into three 8-ml vacutainer cell preparation tubes (CPT; Becton Dickinson) containing each 1 ml of 0.1 M sodium citrate for the SMAD assay. Tubes were inverted five times and centrifuged at room temperature for 25 min in a VanGuard V6500 centrifuge (SmithKline Beecham, PA, USA) at 1300*g*. Following centrifugation, one tube (CPT 1) was shipped for overnight delivery to a central laboratory (Eli Lilly, Indianapolis, IN, USA) on ice packs (Figure 2; Central Stimulation); the other two tubes (CPT 2 and CPT 3) were immediately processed at the site for the *ex vivo* stimulation assay and the untreated PBMC sample (Clinical On-Site Stimulation). Upon arrival at the

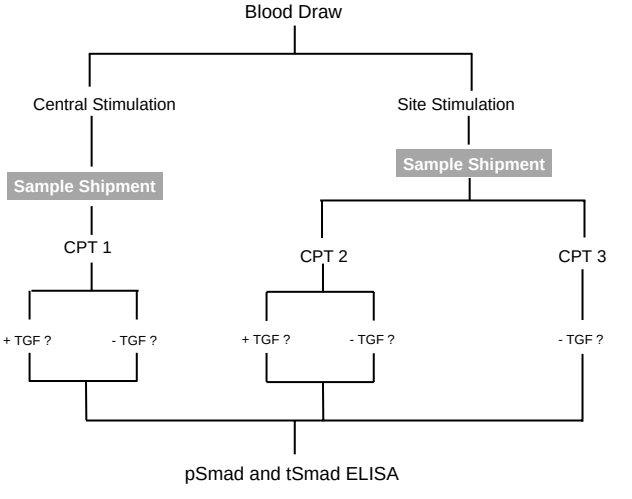

Figure 2. Work flow of the SMAD ELISA assay. Blood was obtained to compare site with central stimulation of peripheral blood mononuclear cells (PBMCs). For stimulation at the central laboratory site, cells from one CPT tube (CPT 1) were split for a TGF-β-stimulated (+ TGF-β) and unstimulated condition (− TGF-β). After the *in vitro* treatment, extracts were prepared to determine phosphorylated (pSMAD) and total (tSMAD) SMAD levels. For the site stimulation assay, two CPT were collected. Cells from CPT tube 2 (CPT 2) were split for a TGF-β-stimulated (+ TGF-β) and unstimulated condition (− TGF-β). The second on-site assessment CPT tube (CPT 3) was not stimulated and used for determining basal levels of pSMAD and tSMAD levels in patients with cancer. After the *in vitro* treatment, extracts were prepared to determine pSMAD and tSMAD levels.

central laboratory, the CPT tubes were processed to measure pSMAD and tSMAD levels in PBMCs, while a similar procedure was first performed at the site prior to shipping the cell lysates for analysis at the central laboratory. CPT tubes delivered to the stimulation laboratory (central or at the site), PBMC were removed from the CPT tube, aliquoted, and treated in the absence or presence of TGF-β1 for 60 min at 37°C. The stimulated or unstimulated PBMCs were washed three times with ice-cold phosphate-buffered saline and lysed in 500 µl of ice-cold lysis buffer containing 1.18% HEPES, 0.188% EDTA, 0.192% EGTA, 0.892% sodium pyrophosphate, 0.872% sodium chloride, 0.084% sodium fluoride, 1% Triton-X 100, 0.0184% sodium orthovanadate, 0.1% bacitracin, 0.0008% leupeptin, 0.008% aprotinin, 0.0182% phenylmethylsulfonyl fluoride, 1X phosphatase inhibitor cocktail I and II (Sigma). The lysates were frozen at -80°C and stored until ELISA analysis was performed using a new ELISA analysis for pSMAD and tSMAD (Farrington et al. 2007).

pSMAD/tSMAD ELISA. The ELISA was optimized to measure both pSMAD and tSMAD (Farrington et al. 2007). Plates were coated by adding 100 µl mouse monoclonal antibody (Becton Dickinson Tranduction Laboratories #S66220) as a tSMAD2/3 capturing antibody at a concentration of 2.5 µg ml^{-1} to each well. The plates were covered and incubated overnight at approximately 4°C. At the completion of every incubation step, the plate was washed five times with 200 µl of Tris-buffered saline made with 0.1% Tween-20 (TBST). Next, the plate was blocked by adding 200 l TBS with 1% bovine serum albumin (BSA) to each well and was incubated at room temperature on rocker for at least 2 h. One hundred microlitres of either sample, standard, or control was added in duplicate to appropriate wells, then covered, and incubated overnight at approximately 4°C. One-hundred microlitres of a rabbit polyclonal antibody raised against CKKS-pS-M-pS-COOH, a multiserine phosphorylated fragment of SMAD2 (custom ordered by Lilly from Zymed, San Francisco, CA, USA), or tSMAD rabbit polyclonal antibody (Zymed #51–1300) diluted to 2.5 µg ml^{-1} in lysis solution with 1 mg ml^{-1} BSA was added to each well of the respective plates. The plates were incubated at room temperature on a rocker for 2 h. Next, 100 l reporter antibody (Amersham #NA934V) diluted 1:10 000 in TBS with 1% BSA was added to each well and incubated at room temperature on rocker for 1 h. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (100 µl) was added to each well and incubated at room temperature on a rocker for 30 min. Then, 100 µl phosphoric acid stop solution was added to each well. The plate was read at 450 λ on Spectromax 384.

Data analysis

Linear mixed effects models were used to estimate the inter- and intrapatient coefficient of variations for pSMAD and TGF-1. The data were log-transformed prior to analysis. The effect of PF4 on TGF-1 concentrations between breast and prostate cancer patients were investigated using both logistic regression, after categorizing patients according to whether they had high or low levels of each analyte, and linear mixed effects models. The non-parametric Spearman rank correlation coefficients were calculated between TGF-1 and each of the downstream biomarkers, including VEGF, PAI-1, IL-11, uPA, PTHrP and the factors in the MAIP. Comparisons between a non-cancer reference population and the cancer patients in this study were carried out for the analytes in the MAIP. This analysis had to take into

account that demographic details for the non-cancer reference population were not available. Therefore, comparisons between the cancer patients on study and the non-cancer reference population were carried out using the simplest of tests, without being able to adjust for factors such as gender or age. In order to obtain an initial understanding of which analytes may prove to be differentially expressed in the two populations, the medians of the two populations for each analyte were compared using the Wilcoxon rank sum method. All analyses involving MAIP controlled for the false discovery rate using the method described by Benjamani and Hochberg (1995).

Results

A total of 49 cancer patients with skeletal metastasis participated in this study conducted at three investigative sites (Vall d'Hebron, Spain; Johns Hopkins, USA; Vanderbilt, USA.). The median age of participating patients was 49 years (range 46–78) and sex was balanced with 28 female and 21 male patients participating in the study. All 49 patients had at least one venipuncture to obtain blood for the biomarker assessments. Breast ($n=23$, 46.9%) and prostate cancer ($n=15$, 30.6%) were the most common tumour types. Of the 49 patients, 23 patients were enrolled at Vall d'Hebron; 21 of these 23 patients had breast cancer. Fifteen of the 49 patients were enrolled at Johns Hopkins; 10 of these 15 patients had prostate cancer. Eleven of the 49 patients were enrolled at Vanderbilt; these patients had various tumour types.

One of the objectives of the study was to determine plasma levels of TGF-β1 in patients with known bone metastasis. Because TGF-β1 can be released by platelet activation after blood draws (Barthelemy-Brichant et al. 2002), we used an approach that can reduce platelet activation prior to blood draws (i.e. release of tourniquet during blood draw) and suppress platelet activation in the collection tube (i.e. use of CTAD tubes). About 70% of all the patients had elevated TGF-β1 levels in this study (Figure 3) with a mean plasma TGF-β1 level of 2.63 ng ml^{-1} (95% confidence interval (CI) 2.32–2.98. and PF4 levels of 31.4 IU ml^{-1} (95% CI 24.4–40.5), averaged across visits (Figure 3A, B; open symbols denote samples with no elevated PF4, while closed symbols are samples with elevated PF4 levels). Most patients from Vall d'Hebron were breast cancer patients and had lower PF4 values. By contrast, patients from Johns Hopkins had mostly prostate cancer and concomitant elevation of PF4 and TGF-β1. Vanderbilt enrolled patients with the highest TGF-β1 and PF4 levels (Figure 3A). In addition, patients with multiple myeloma had also high TGF-β1 and PF4 levels (Figure 3B). After adjusting for the correlation between TGF-β1 and PF4, TGF-β1 concentrations in patients with prostate cancer are on average 1.4 times higher (95% confidence limits 1.2, 1.7) than in patients with breast cancer. Corresponding analyses identified that there was no evidence of a difference in PF4 concentrations between the two indications, after adjusting for the relationship with TGF-β1.

While plasma TGF-β1 levels can be important to identify patients with an active TGF-β-dependent tumour growth, measuring downstream proteins of TGF-β signalling offers an additional approach to understand TGF-β-dependent tumour progression. We measured levels of PAI-1, VEGF, uPA, IL-11 and PTHrP in samples collected on visit 1 (Figure 1) using a validated assay provided by Pathway Diagnostics. We found that only PTHrP was correlated with plasma TGF-β1 levels ($r=0.34$; $p<0.05$) (Figure 4), although it should be noted that at least half the patients had undetectable levels of PTHrP. Plasma IL-11 levels were below the limit of

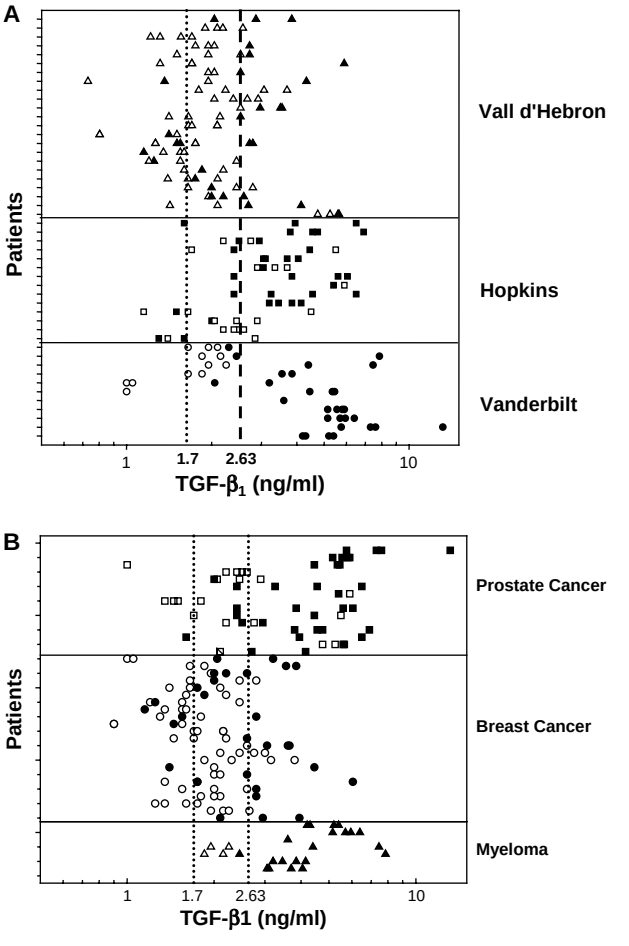

Figure 3. (A) Plasma TGF-β1 levels in patients with skeletal metastasis. Each tick mark on the *y*-axis represents a patient with up to four consecutive blood draws (left *y*-axis). Each site (triangle, Vall d'Hebron; square, Hopkins; circle, Vanderbilt) participating in the study is shown to allow a comparison of site-to-site variability for the ELISA assay of plasma TGF-β1 (depicted in log scale on the *x*-axis, with normal value indicated at 1.7 ng ml^{-1} as dotted line). The geometric mean averaged across all visits was 2.63 ng ml^{-1} (dashed line). To control the influence of possible platelet activation to increase the plasma TGF-β1 levels, platelet factor 4 (PF4) was assessed at each measurement of plasma TGF-β1. Open symbols represent patients with normal PF4 levels (<35 IU ml^{-1}) and closed symbols represent samples with elevated PF4 levels (>35 IU ml^{-1}). Patients with the highest number of elevated PF4 levels and high plasma TGF-β1 were present at Vanderbilt, while those with the lowest were observed at Vall d'Hebron. The intrapatient variability was 37% CV and the interpatient variability was 57% CV. (B) In addition to comparing assay variability due with multisites, we also compared biological variability. Average TGF-β1 concentrations for prostate and breast cancer patients were 3.19 ng ml^{-1} and 2.25 ng ml^{-1}, respectively. In addition prostate cancer patients appeared to have more $PF4^{+}$/TGF-$β1^{+}$ levels than breast cancer patients (3x more likely to have raised levels in both analytes; $p<0.01$). Interestingly, patients with multiple myeloma (triangles) also had elevated TGF-β1 and PF4 levels.

detection in all 49 patient samples. No data from a reference population were available and therefore a comparison with this cancer population could not be made.

In contrast to standard ELISA, novel fluorocytometric-based immunoassays can offer a different approach to complex biomarker investigation. We here tested for the

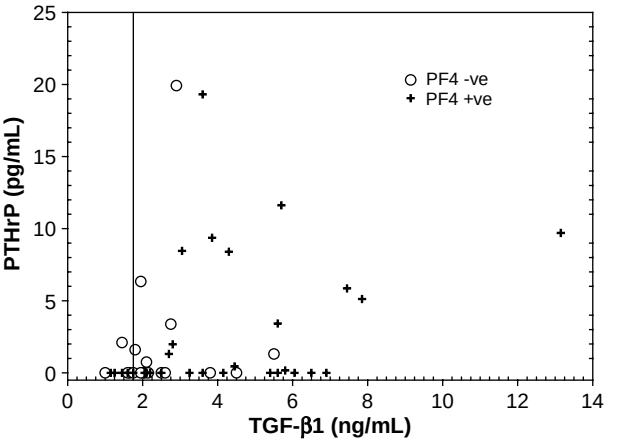

Figure 4. Comparison of plasma TGF-β1 levels with PTHrP. Open circles represent samples with TGF-$\beta1^+$/$PF4^-$ measurements, while + symbols stand for TGF-$\beta1^+$/$PF4^+$ samples. A vertical line is drawn to indicate normal plasma TGF-β1 levels at 1.7 ng ml^{-1}. Undetected levels of PTHrP were ascribed a value of zero (the LLQ) prior to analysis.

first time a multianalyte immunoassay panel (MAIP) provide by RBM to measure 79 different proteins simultaneously in plasma of cancer patients (Table I). Of these 79 analytes, 53 were detected as being elevated (Table I, Group A–C) and three as being lowered (Table I Group D) in the present study population at the 5% false discovery rate, i.e. three of the 56 have been falsely identified as being differently expressed. Because we used this panel for the first time in cancer patients, we assessed whether the included tumour markers appropriately recognized the relevant tumour type. As expected prostate-specific antigen (PSA) levels were only elevated in patients with prostate cancer (Figure 5A), while other cancer patients had no increased PSA levels. Using all 49 patients for the other tumour markers, medians in the cancer population compared with those in the reference population provided by RBM (Figure 5A, B) were increased for $_2$-microglobulin, CA 125, α-fetoprotein (AFP) and carcinoembryonic antigen (CEA). Other markers, such as ENA-78, were not elevated in the study population or in specific tumour types (Figure 5B). Another assessment to confirm the usefulness of the MAIP was performed on comparing the measurements for two analytes that were assessed by standard ELISA techniques and in the MAIP (Figure 6). In the MAIP, PAI-1 was slightly higher in the study population compared with the reference (Figure 6A). When we correlated PAI-1 and VEGF with the standard ELISA from Pathway Diagnostics, only PAI-1 showed a strong correlation between the MAIP and the standard ELISA measurements (Figure 6B). For the VEGF we did not observe a correlation (data not shown), perhaps due to the difference between the antibody pair used in the standard ELISA compared with the MAIP.

After confirming that the MAIP was a tool to determine multiple secreted proteins in cancer patients, we then correlated TGF-β1 plasma levels with analytes from the MAIP. Prior to adjusting the significance of the correlations for multiple testing, two analytes showed a significant negative correlation and 20 showed significant positive correlations with plasma TGF-β1 levels based on the 0.1 significance level. After adjusting for multiplicity (Table I, last column), only five remained significantly correlated with TGF-β1 at the 10% false discovery rate. The most likely analytes to be

Table I. MAIP markers classified into 4 groups (A–D) after analysis comparing cancer population with reference population and identifying those markers with some evidence of association with TGF-β1 plasma levels.

	Reference population[a]			Study population			
Groups of MAIP markers	Median	5th percentile	95th percentile	Median cancer	Percentile of reference population	Correlation between reference and study population (FDF *p*-value from Wilcoxon rank sum)	Correlation between TGF-β1 and MAIP markers (FDR *p*-value of Spearman rank correlation with TGF-1)
Group A. Markers that are significantly elevated and correlated with TGF-β1							
von Willebrand Factor (μg ml^{-1})	21.150	5.070	70.400	36.90	80–85	0.0000	0.0711
Interleukin (IL)-10 (pg ml^{-1})	7.433	1.560	27.000	15.40	85–90	0.0000	0.0853
C-reactive protein (μg ml^{-1})	2.560	0.170	27.400	8.20	80–85	0.0000	0.0853
Prostatic acid phosphatase (ng ml^{-1})[c]	0.176	0.058	0.576	0.24	75	0.0008	0.0853
Adiponectin (μg ml^{-1})	4.800	1.580	12.800	1.84	45–50	0.4678	0.0853[b]
Group B. Markers that are significantly elevated for cancer population compared with reference population							
Calcitonin (pg ml^{-1})	4.733	0.487	16.400	6.65	85–90	0.0000	0.5282
Cancer antigen 125 (U ml^{-1})	3.878	0.890	11.585	9.36	>95	0.0000	0.3600
Glutathione S-transferase (ng ml^{-1})	0.692	0.264	2.880	1.58	90–95	0.0000	0.9593
IL-12 p40 (ng ml^{-1})	0.618	0.185	1.500	0.74	90–95	0.0000	0.2861
IL-6 (pg ml^{-1})	17.850	6.950	25.000	20.40	90–95	0.0000	0.2625
MMP-3 (ng ml^{-1})	0.395	0.081	1.950	1.84	>95	0.0000	0.9593[b]
IL-1β (pg ml^{-1})	3.070	0.855	8.780	6.25	90–95	0.0000	0.3600
Lymphotactin (ng ml^{-1})	0.161	0.016	0.392	0.33	90–95	0.0000	0.4187
IL-2 (pg ml^{-1})	21.250	7.800	58.450	25.60	85–90	0.0000	0.9593
IL-1α (ng ml^{-1})	0.106	0.019	0.307	0.15	85–90	0.0000	0.4469
IL-3 (ng ml^{-1})	0.150	0.020	0.958	0.46	90–95	0.0000	0.9593
Alpha-fetoprotein (ng ml^{-1})	2.210	0.327	7.050	4.39	85–90	0.0000	0.2475
IL-12 p70 (pg ml^{-1})	44.100	15.250	104.500	71.60	85–90	0.0000	0.3600
IL-5 (pg ml^{-1})	13.720	2.475	46.950	26.70	85–90	0.0000	0.6424
IL-8 (pg ml^{-1})	8.143	1.645	90.267	14.20	80–85	0.0000	0.2861
Stem cell factor (pg ml^{-1})	56.000	2.680	267.000	99.20	80–85	0.0000	0.5436
TNF-β (pg ml^{-1})	21.050	5.000	62.400	26.60	85	0.0000	0.5472
IL-4 (pg ml^{-1})	26.058	11.100	79.750	51.30	85–90	0.0000	0.9934
IL-13 (pg ml^{-1})	30.850	0.640	108.000	55.70	80–85	0.0000	0.4578[b]
FGF basic (pg ml^{-1})	69.667	20.450	142.500	39.90	80–85	0.0000	0.5436

Table I (*Continued*)

Groups of MAIP markers	Reference population[a]			Study population		Correlation between reference and study population (FDF *p*-value from Wilcoxon rank sum)	Correlation between TGF-β1 and MAIP markers (FDR *p*-value of Spearman rank correlation with TGF-1)
	Median	5th percentile	95th percentile	Median cancer	Percentile of reference population		
GM-CSF (pg ml^{-1})	25.117	4.040	72.900	52.80	90	0.0000	0.6424
MIP-1β (pg ml^{-1})	71.667	25.000	433.500	150.00	85	0.0000	0.9234
TNF-α (pg ml^{-1})	5.418	1.405	25.900	8.81	80–85	0.0000	0.2861
Endothelin-1 (pg ml^{-1})	4.935	2.205	9.350	4.44	80–85	0.0000	0.3600
IL-15 (ng ml^{-1})	1.039	0.095	2.450	1.86	85–90	0.0000	0.3600
IL-18 (pg ml^{-1})	179.500	63.767	1,255.000	275.00	80–85	0.0000	0.5282
MCP-1 (pg ml^{-1})	93.067	29.750	335.000	167.00	80–85	0.0000	0.9593[b]
TNF RII (ng ml^{-1})	78.300	48.933	221.000	109.80	85	0.0000	0.2599
Growth hormone (ng ml^{-1})	0.329	0.021	4.650	0.64	75–80	0.0000	0.9593[b]
Ferritin (ng ml^{-1})	78.300	4.380	508.000	1176.00	75–80	0.0000	0.1433
IL-7 (pg ml^{-1})	33.400	3.670	116.000	54.80	70–75	0.0000	0.8128
Creatine kinase-MB (ng ml^{-1})	0.375	0.059	1.430	0.06	80–85	0.0000	0.9593
Serum amyloid P (μg ml^{-1})	27.200	14.433	45.600	35.00	85–90	0.0000	0.3537
TIMP-1 (ng ml^{-1})	95.667	55.000	201.000	131.00	80	0.0000	0.2625
β_2-Microglobulin (μg ml^{-1})	1.843	1.130	3.717	2.31	75–80	0.0000	0.3128
α_2-Macroglobulin (mg ml^{-1})	0.207	0.133	1.593	0.25	75–80	0.0002	0.2861[b]
MMP-9 (ng ml^{-1})	169.000	2.885	883.000	244.80	65–70	0.0002	0.3733
SGOT (μg ml^{-1})	13.900	3.563	28.250	18.40	65–70	0.0002	0.3600[b]
Insulin (uIU ml^{-1})	4.120	0.106	33.750	6.94	70–75	0.0004	0.4142
Fibrinogen (mg ml^{-1})	4.020	2.093	7.490	4.62	65–70	0.0005	0.5282
IL-16 (pg ml^{-1})	487.500	215.000	3,190.000	644.00	70–75	0.0005	0.3824
Thrombopoietin (ng ml^{-1})	2.425	0.101	6.010	2.94	65–70	0.0011	0.9593
α_1-Antitrypsin (mg ml^{-1})	1.643	1.090	2.493	4.61	70–75	0.0013	0.5436
Eotaxin (pg ml^{-1})	32.975	11.950	160.000	41.00	70–75	0.0035	0.5844[b]
Thyroxine binding globulin (μg ml^{-1})	66.567	39.333	105.500	72.80	65	0.0118	0.9593[b]
PAI-1 (ng ml^{-1})	29.775	8.580	83.200	36.30	65–70	0.0129	0.2625
Myoglobin (ng ml^{-1})	7.400	3.353	25.667	11.05	70–75	0.0193	0.9412[b]

Table I (*Continued*)

Groups of MAIP markers	Reference population[a] Median	5th percentile	95th percentile	Study population Median cancer	Percentile of reference population	Correlation between reference and study population (FDF *p*-value from Wilcoxon rank sum)	Correlation between TGF-β1 and MAIP markers (FDR *p*-value of Spearman rank correlation with TGF-1)
Complement 3 (mg ml^{-1})	1.270	0.764	2.235	1.39	60–65	0.0202	0.3600
Carcinoembryonic antigen (ng ml^{-1})	1.585	0.255	5.663	1.60	50–55	0.0375	0.6565[b]
Group C. Markers not significantly different for cancer population compared with reference population							
Leptin (ng ml^{-1})	8.677	0.384	40.600	11.75	65–70	0.0773	0.4187[b]
Fatty acid binding protein (ng ml^{-1})	3.080	0.276	10.110	3.82	55–60	0.0894	0.8789
MMP-2 (ng ml^{-1})	360.500	183.667	843.667	428.50	60–65	0.1094	0.4187[b]
VCAM-1 (ng ml^{-1})	495.667	266.667	986.000	537.00	55–60	0.1143	0.5436
Tissue factor (ng ml^{-1})	1.100	0.404	2.520	0.40	40	0.1154	0.8339[b]
Lipoprotein a (μg ml^{-1})	55.200	2.990	835.000	81.90	55–60	0.1540	0.9593[b]
Cancer antigen 19-9 (U ml^{-1})	0.930	0.044	6.040	0.61	40–45	0.2537	0.9593
Brain-derived neurotrophic factor (ng ml^{-1})	1.890	0.349	18.250	2.27	50–55	0.3479	0.9243
Apolipoprotein A1 (mg ml^{-1})	0.395	0.179	0.898	0.40	50–55	0.3902	0.5282[b]
EGF (pg ml^{-1})	24.250	1.050	175.000	5.48	50–55	0.4121	0.2861
ICAM-1 (ng ml^{-1})	103.000	39.133	193.667	106.00	55	0.4678	0.5584
MDC (pg ml^{-1})	342.000	119.667	625.000	364.00	55–60	0.5881	0.6151
MIP-1α (pg ml^{-1})	17.125	2.370	62.600	11.80	45–50	0.5881	0.9166[b]
RANTES (ng ml^{-1})	14.500	2.590	82.650	13.80	45–50	0.6886	0.5282
Apolipoprotein H (μg ml^{-1})	266.000	119.000	425.000	286.50	60–65	0.7682	0.9166
Erythropoietin (pg ml^{-1})	83.650	48.250	124.000	48.30	50	0.7841	0.3254
Factor VII (ng ml^{-1})	231.500	95.900	413.333	241.50	50–55	0.8320	0.2861
Prostate-specific antigen, free (ng ml^{-1})[c]	0.076	0.023	0.805	0.04	40–45	0.8320	0.2301
Thyroid stimulating hormone (μIU ml^{-1})	1.015	0.154	3.360	0.92	40–45	0.8320	0.5652[b]
VEGF (pg ml^{-1})	223.000	89.600	842.000	210.50	45–50	0.8320	0.6036
ENA-78 (ng ml^{-1})	0.559	0.062	4.997	0.55	45–50	0.8568	0.5282
Apolipoprotein CIII (μg ml^{-1})	74.700	28.400	236.000	72.60	45–50	0.8683	0.5870

Table I (*Continued*)

Groups of MAIP markers	Reference population[a]			Study population		Correlation between reference and study population (FDF *p*-value from Wilcoxon rank sum)	Correlation between TGF-β1 and MAIP markers (FDR *p*-value of Spearman rank correlation with TGF-1)
	Median	5th percentile	95th percentile	Median cancer	Percentile of reference population		
Group D. Markers that are significantly lower in cancer population compared with reference population							
IgE (ng ml^{-1})	59.100	1.760	1,140.000	2.63	5–10	0.0000	0.1433
IgM (mg ml^{-1})	0.935	0.277	3.265	0.61	20–25	0.0000	0.3600[b]
IgA (mg ml^{-1})	2.453	0.658	5.750	1.93	30–35	0.0127	0.6565

[a]Reference range are approximated by the 5th and 9th percentiles from the ~250 subjects' data for each analyte provided by Rules Based Medicine. [b]Analytes where the Spearman Rank correlation coefficient was negative. [c]These comparisons are based on all cancer patients assessed during the study. The medians for the 15 prostate cancer patients were 11.5 and 1.38 for PSA and PAP, respectively, clearly different from the non-cancer population (>95th percentile of distribution of reference population in both cases).

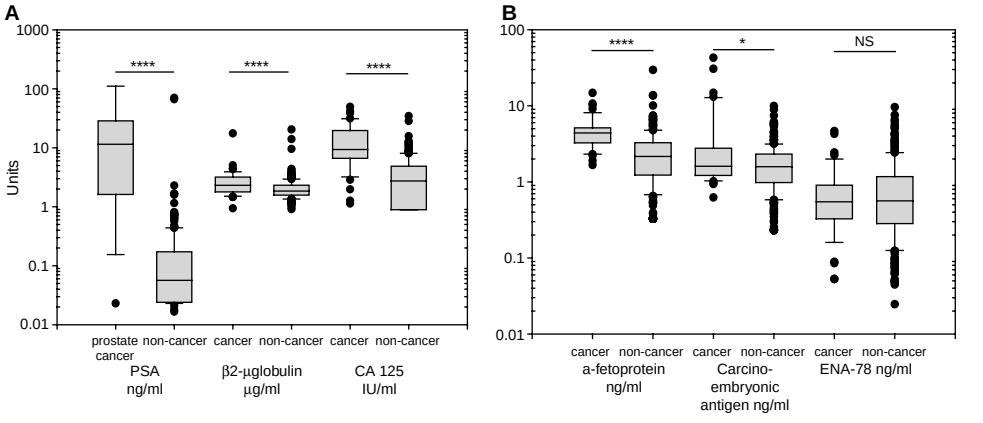

Figure 5. Plasma concentrations of established tumour markers and ENA-78 using the multianalyte immunoassay panel (MAIP, Rules Based Medicine). (A, B) The plasma concentrations for PSA, β_2-microglobulin, CA125, α-fetoprotein, CEA and ENA-78. Cancer patients refer to the patients enrolled in the present study, while non-cancer denotes the reference population with no known malignancies ($n = \sim 250$; provided by Rules Based Medicine). For PSA, only patients with known prostate cancer were included to compare with the reference population. Each box represents the median and 25th and 75th percentiles of the distribution for each sample; the error bars, the 5th and 95th percentiles. Significance levels: ****$p < 0.0001$; *$p < 0.05$; NS, not significant. (To aid interpretation of the significance levels, please refer to Table I to determine which percentile of the reference population the median of the cancer population compares with.)

positively correlated with plasma TGF-β1 were vWF, C-reactive protein (CRP), IL-10 and prostatic acid phosphatase (PAP). No correlation was observed between PF4 positive samples and vWF elevation.

Finally, we assessed whether the plasma TGF-β1 levels correlated with activation of its pathway in PBMCs by assessing SMAD expression (Figure 2). Before we applied this assessment in PBMCs of patients, specific details of the method needed to be optimized for generating reproducible results. Using a novel ELISA to quantify pSMAD and tSMAD levels, we detected no pSMAD or tSMAD levels in samples obtained directly from patients (CPT3 in Figure 2; data not shown). This lack of a baseline detection for pSMAD led to the use of an *ex vivo* stimulation assay (Farrington et al. 2007), which subsequently could be applied in clinical trials to determine activity of TGF-β inhibitors. One aspect of testing this *ex vivo* stimulation assay was to determine whether samples must be stimulated immediately at the site, or if samples could be shipped and stimulated the following day at a central laboratory (Figure 2). To determine which condition provided the most reproducible results, two blood samples were drawn from each patient in CPT tubes (CPT 1 and CPT 2); one tube was submitted to the central laboratory and the other was treated at the site until cell lysates were obtained. Vall d'Hebron was the only site of the three that could not participate in the overnight shipment of this part of the study, because a 24-h shipment to the USA was not feasible. All cell lysates were used to determine pSMAD and tSMAD levels by ELISA. The pSMAD levels (adjusted for tSMAD) of each patient sample stimulated at the site at each visit are shown in Figure 7A and B. All samples exposed to *ex vivo* TGF-β1 stimulation had an increase in pSMAD levels regardless of where the treatment was performed. The pSMAD levels (adjusted for tSMAD) were also sorted by the patient's tumour type (Figure 7B). No differences in pSMAD levels were observed based on tumour type. To determine the best condition

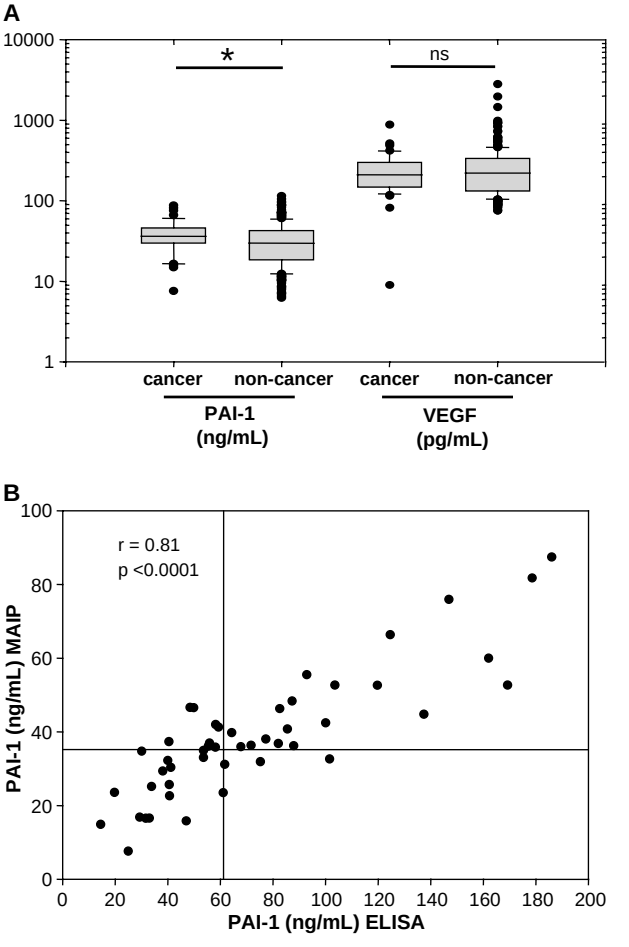

Figure 6. Comparison between standard ELISA and RBM panel for PAI-1 and VEGF. (A) The differences between cancer and non-cancer patients for PAI-1 and VEGF from MAIP. (B) The correlation between PAI-1 ELISA results (Pathway) and MAIP (Spearman rank correlation = 0.81; $p < 0.0001$). For both analytical methods, the medians are depicted as a vertical (ELISA) and horizontal line (MAIP). VEGF is not shown because of a lack of correlation due to differences in antibodies used in both analytical methods.

for performing the *ex vivo* stimulation with TGF-β1, the inter- and intrapatient variability were estimated for the samples stimulated at the site and the samples stimulated at the central laboratory. The intrapatient coefficient of variation for pSMAD was estimated as 47% (95% CI 42–55%) when the stimulation was performed at the site and 54% (95% CI 42–76%) when the stimulation was performed at Lilly's central laboratory. The results from these analyses indicate that intrapatient variability is likely to be lower if samples are stimulated immediately at the investigator sites prior to shipping.

Discussion

Because of the shift in clinical development towards target-based therapies in cancer (Fox et al. 2002), TGF-β inhibitors may be a useful treatment option for patients

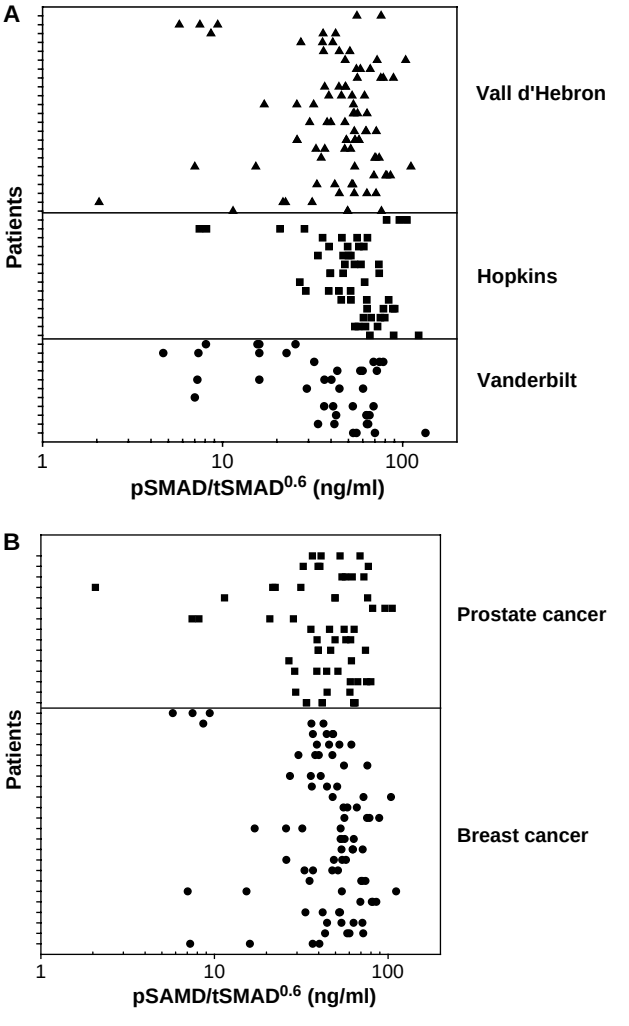

Figure 7. pSMAD expression in PBMCs of patients after *ex vivo* stimulation with TGF-β at three different sites (triangle, Vall d'Hebron; square, Hopkins; circle, Vanderbilt). pSMAD expression of each patient is plotted on separate lines. Each line contains measurements of pSMAD normalized to total SMAD levels (tSMAD) at the four visits of each patient and intrapatient coefficients of variation are reported by site (A) and by tumour type (B). Interpatient CV% was 79% and intrapatient CV% was 47%.

with an active TGF-β signalling pathway causing tumour growth. To be successful in this target-based approach in cancer, biomarkers of activity are necessary in the early clinical studies. Examples of the usefulness of such biomarker-based development strategies have been applied for the development of mTOR inhibitors (Boulay et al. 2004) and also for anti-angiogenic compounds, such as SU11248 (O'Farrell et al. 2003, Boulay et al. 2004). In both of these examples, drug exposure was related to a specific target inhibition measured in cells obtained from the blood of patients. Recently, technology of multiplexing provides the ability to concurrently assess tumour-associated plasma markers and connect drug response to a profile of plasma markers of response (Zeh et al. 2005). Similarly to the established tumour

markers, such as CA19-9 for pancreatic cancer, a serum or plasma marker that demonstrates a relationship between TGF-β and tumour growth might enhance the clinical development of TGF-β inhibitors. Limiting this study to the participation of patients with skeletal metastasis was based on the observation that TGF-β signalling is increased in patients with bone metastasis, and hence providing a hypothetically enriched patient population for increased TGF-β signalling (Kang et al. 2005). We found that half of the patients had consistently elevated TGF-β1 levels confirming that bone metastases are possibly associated with elevated TGF-β1 levels. Interestingly, a number of patients with elevated TGF-β1 levels also had elevated PF4 levels. While increased PF4 levels may be a result of platelet activation during blood collection, elevated PF4 can also reflect pathophysiological processes in patients. For instance, PF4 was first found to be elevated in patients with coronary artery disease and used as a measure of *in vivo* platelet activation (Levine et al. 1981). In cancer patients, PF4 elevation associated with *in vivo* platelet activation has also been observed (Wynendaele et al. 1999). Whether this activation is caused by or correlated with the well-recognized health problem of hypercoagulation in patients with metastatic disease (Caine et al. 2002) remains to be determined. Other causes that may explain the elevation of PF4 in the cancer patients may include mechanisms similar to those found in patients with severe inflammatory and pulmonary diseases (Buyukasik et al. 1998, Kowal-Slungaard 2004, Bielecka et al. 2005). Hence, ascribing PF4 elevations solely to platelet activation after blood draws is too simplistic. While in the present study we did not compare TGF-β1 and PF4 in patients with and without skeletal metastasis, our study provides a first assessment on the expected patient number likely to show elevated levels of TGF-β1 and PF4. Such an assessment is helpful in estimating the patient number required to document the potential effect of TGF-β inhibitors on TGF-β levels in patients with skeletal metastasis. This focus on particular types of patient is clinically practical and consistent with the enrolment of patients in early-phase oncology clinical trials. Moreover, it appeared that TGF-β1 levels may be different between the various tumour types despite the fact that all patients had documented skeletal metastasis (Figure 3). To determine whether this difference is cancer-related and not attributable to variable processing at the different sites, future studies should include control sampling from healthy donors at each of the clinical sites.

Furthermore, downstream proteins generally associated with TGF-β1 levels, such as uPA, PTHrP and IL-11, were assessed in this study (Siegel & Massague 2003). With the exception of PTHrP (Kakonen et al. 2002), the other markers were not correlated with TGF-β1. This observation is remarkable as PTHrP has been associated with skeletal metastasis and with hypercalcaemia (Rizzoli et al. 1999). Thus, this observation fits with the previous hypothesis that TGF-β activation is an important part of the skeletal metastasis and connected to PTHrP activation (Kang et al. 2003).

In addition to evaluating the correlation of plasma TGF-β1 levels with secreted proteins of known association (e.g. PTHrP, uPA), the use of proteomic analytic tools (e.g. MAIP) can identify unappreciated correlations between TGF-β1 and other secreted proteins or markers. To detect such unappreciated correlations, we used MAIP from Rules Based Medicine which is designed to detect 79 different markers. This particular panel can be grouped into three different categories: (1) inflammation-related markers (e.g. CRP, IL-10, IgE, sTNFRII, IL-6, IL-8, IL-12p40,

TNF-α, serum amyloid P); (2) angiogenesis-related markers (e.g. vWF, TIMP-1, PAI-1, EGF); (3) tumour markers and other markers (e.g. PAP, ferritin, PSA, AFP, factor VII, β_2-microglobulin, erythropoietin). Concurrent assessment of TGF-β1 levels with the other markers of the MAIP was not possible and hence a correlation between TGF-β1 levels and the MAIP markers was based on the TGF-β1 values of plasma obtained from CTAD tubes with the EDTA-derived MAIP markers. Future studies will have to determine whether using CTAD tubes for MAIP markers will result in different measurements compared with EDTA plasma-based evaluations. Recent studies in animals suggest that there is no difference in results if plasma or serum-derived samples are being used to assess MAIP markers (personal communication from Rules Based Medicine). Thus, it is not likely that EDTA or CTAD plasma will result in significant changes in MAIP markers. The MAIP identified previously unappreciated correlations (after adjustment for multiplicity) between TGF-β1 levels and vWF, IL-10, CRP, prostatic acid phosphatase and adiponectin (Table I, Group A). Because the MAIP is a novel tool, the comparison with the reference population is limited and additional confirmatory studies will be needed to interpret the importance of this detection tool for biomarker discovery efforts. Of these 79 analytes, 53 were found to be elevated compared with the reference population provided by RBM suggesting that a large number of secreted proteins were increased in this cancer population (Table I, Groups A and B). Other markers of MAIP were not elevated in cancer patients compared with the reference population (Table I, Group C). None of the markers in either of these groups (Table I, Groups B and C) were significantly correlated with TGF-β1 levels.

Among those markers with the strongest correlation with TGF-β1 levels were vWF and IL-10. vWF is a protein which facilitates the adhesion of platelets to the endothelial cells and thus functions as an important factor in wound healing (Matsushita et al. 1994). While its secretion is generally associated with activated platelets, high levels have also been observed in breast cancer patients where it correlates with stage of disease (Rohsig et al. 2001). Similarly to TGF-β, vWF is associated with increased neo-angiogensis and can be used as a measure for determining the degree of vascular density in a tumour (Vincent-Salomon et al. 2001). In both these instances, the presence in the plasma and its expression in the tumour are associated with poor clinical outcome. Interestingly, IL-10 was associated with TGF-β1 levels. Recent animal studies support that both IL-10- and TGF-β-secreting T cells are required to suppress anti-tumour immunity (Jarnicki et al. 2006). This concept is also currently being evaluated for diagnostic application, in which the anti-tumour activity is being measured by concomitantly following TGF-β and IL-10 levels after treatment (Wojtowicz-Praga 1997).

Another aspect of this study was to determine whether the presence of circulating and elevated TGF-β1 was associated with SMAD expression in PBMCs. A correlation between tSMAD and TGF-β1 levels was not convincingly found in this study. Also, we could not detect pSMAD in PBMC of patients with high plasma TGF-β1 levels. This lack of correlation between either tSMAD or pSMAD with high TGF-β1 levels is probably due to the sensitivity of the ELISA used in this study.

Since pSMAD levels were undetectable, we evaluated an *ex vivo* assay for pSMAD measurements. As with other *ex vivo* assays (Green et al. 2006), variability can increase as a result of sample collection and time to processing – either at the site or at a central laboratory. We compared both approaches and found that the least variable

result was obtained when PBMCs were stimulated and processed immediately. The pSMAD intrapatient variability was 47% and may be sufficiently robust to determine changes induced by TGF-β inhibitors in patients.

In conclusion, we have observed that TGF-β1 levels are elevated in patients with advanced, metastatic disease and skeletal metastasis. The TGF-β1 levels in these patients correlate with PTHrP, vWF, CRP and IL-10. These markers may help in assessing the activity of future TGF-β-inhibitor studies in patients. Finally, we established an *ex vivo* assay which can be applied to dose-finding studies of TGF-β inhibitors during the clinical development of a TGF-β receptor kinase inhibitor.

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