RESEARCH PAPER

Serum follistatin in patients with prostate cancer metastatic to the bone

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Abstract The clinical significance of circulating follistatin (FLST), an inhibitor of the multifunctional cytokine activin A (Act A), was investigated in patients with prostate cancer (PCa). The serum concentrations of this molecule were determined by an enzyme-linked immunosorbent assay (ELISA) in PCa patients with (M+) or without (M0) bone metastases, in patients with benign prostate hyperplasia (BPH) and in healthy subjects (HS). The effectiveness of FLST in detecting PCa patients with skeletal metastases was determined by the receiver operating characteristic (ROC) curve analysis. Serum FLST was significantly higher in PCa patients than in BPH patients ($P = 0.001$) or HS ($P = 0.011$). Conversely, in BPH patients, FLST levels resulted lower than in HS ($P = 0.025$). In cancer patients the serum concentrations of FLST significantly correlated with the presence of bone metastases ($P = 0.0005$) or increased PSA levels ($P = 0.04$). Interestingly, significant differences in the ratio between FLST and Act A serum concentrations (FLST/Act A) were observed between HS and BPH patients ($P = 0.001$) or PCa patients ($P = 0.0005$). Finally, ROC curve analysis, highlighted a sound diagnostic performance of FLST in detecting M+ patients ($P = 0.0001$). However, the diagnostic effectiveness of FLST did not result significantly superior to that of Act A or PSA. These findings suggest that FLST may be regarded as a potential, molecular target in the treatment of metastatic bone disease while its clinical role as soluble marker in the clinical management of PCa patients with bone metastases needs to be better defined.

Keywords Activin · Bone metastasis · Cancer · Follistatin · Prostate cancer · Transforming growth factor β · Tumor markers

Abbreviations

Act A	Activin A
ActRII	Activin type II receptor
AUC	Area under the curve
BMP-7	Bone morphogenic protein-7
BPH	Benign prostate hyperplasia
FLST	Follistatin
FLST/Act A ratio	Follistatin/Activin A ratio
ELISA	Enzyme linked immunosorbent assay
HS	Healthy subjects
MMP-2	Matrix-metalloproteinase-2
M0	Patients with confined disease
M+	Patients with bone metastases
PCa	Prostate cancer
PSA	Prostate specific antigen
ROC curve	Receiver operating characteristic curve
SCLC	Small cell lung cancer
TGF-β	Transforming growth factor-beta
VEGF	Vascular endothelial growth factor

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Introduction

Activin A (Act A) is a multifunctional cytokine of the transforming growth factor-beta (TGF-β) superfamily of growth factors [1]. This molecule appears to be implicated in the regulation of a broad range of important biological processes including cell proliferation, differentiation, apoptosis, immune response, wound healing, embryogenesis, endocrine functions, tissue metabolism and homeostasis [1–3]. The biological activity of activin A is modulated by several endogenous inhibitors including follistatin (FLST), a cystein rich glycosylated polypeptide chain of 31–39 kDa that binds Act A with high affinity (850–500 pM) [1, 3, 4]. Interestingly, growing evidence suggests that the activin–follistatin system (Act/FLST) may have a regulatory function in controlling several physiological and pathological processes [3, 5, 6]. In particular, recent findings indicate that these molecules appear to have a role in the regulation of bone turnover in normal and pathological conditions. To confirm this, several experimental studies provide evidence that the Act/FLST system modulates osteoclast activity, osteoblast formation, cartilage maturation and endochondral bone formation [6–13] and that the balance between these molecules is deregulated in several disorders associated with increased bone degradation [6, 9, 14, 15]. These observations might suggest the possible involvement of FLST, in particular, in the pathogenesis of bone metastasis, a pathological process which is known to be associated with marked disturbances of bone turnover [16]. This hypothesis is corroborated by many observations showing that tumors which preferentially metastasize to the bone including prostate cancer present altered expression levels of FLST [3, 17–26]. Furthermore, the possible correlation between the diffusion of malignant cells into the bone and alteration in the Act A/FLST balance in patients with prostate cancer is further indirectly supported by certain recent studies of ours which demonstrate that the circulating levels of Act A are markedly elevated in these patients and that this phenomenon correlates with the presence and number of skeletal metastases [27]. On the basis of these findings we have undertaken further investigation in order to assess the clinical significance of circulating FLST in PCa patients with bone metastasis and its relationship with Act A.

Patients and methods

These studies were carried out on serum samples, collected between 2004 and 2006, from 35 patients with prostate cancer, 20 patients with histologically proven benign prostatic hyperplasia (BPH) (median age 61.9, range 55–74) and 21 registered male healthy blood donors (HS)

Table 1 Correlation between follistatin serum concentrations and some clinical and biological parameters of PCa progression

Parameter	Median follistatin (ng/ml) and (range)	P value
Gleason score		
2–4 (n = 11)	1.9 (0.9–5.9)	
5–7 (n = 15)	1.7 (0.66–4.5)	
8–10 (n = 8)	2.8 (1.0–8.1)	0.11 (n.s.)[a]
Extracapsular extension		
Negative (n = 5)	2.0 (0.83–5.9)	
Positive (n = 7)	1.9 (1.5–8.1)	0.88 (n.s.)[b]
Lymph node status		
Negative (n = 7)	3.8 (0.83–8.1)	
Positive (n = 9)	1.9 (1.2–4.5)	0.07 (n.s.)[c]
Bone metastasis		
M0 (n = 15)	1.7 (0.66–3.8)	
M+ (n = 20)	2.7 (1.25–8.1)	0.0005[b]
PSA		
<17.0 ng/ml (n = 14)	1.8 (0.66–4.2)	
>17.0 ng/ml (n = 20)	2.9 (1.25–8.1)	0.04[b]

Results are reported as median values and (range). Due to some missing sample, number of subjects in each group may vary. In *parenthesis*, number of patients

[a] Kruskall–Wallis test; [b] Mann–Whitney *U* test. [c] Student *t* test for independent samples

n.s. Not significant

who served as control group. The prostate cancer group (PCa) comprised 15 patients with confined disease (M0) and 20 patients with clinically documented bone metastases and no apparent extraskeletal involvement (M+) [27]. The main characteristics of cancer patients are reported in Table 1.

Determination of follistatin serum concentrations

Peripheral venous blood specimen from PCa patients or BPH patients were collected before starting any clinical treatment. The blood was drawn into polycarbonate tubes, allowed to clot at room temperature and then centrifuged at 3,500 rpm for 15 min (Hereus Omnifuge 2.0 RS, Hereus Sepatech). Serum aliquots were stored at −80°C until assays were performed. Total FLST serum concentrations (i.e., FS288, FS300 and FS315 isoforms) was determined by a commercially available two-step sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D System, Minneapolis USA) in accordance with the manufacturer's instructions. The reported detection limit was 29 pg/ml (range 10–83 pg/ml). According to the manufacturer, FLST does not cross-react with recombinant human activin A, activin B and activin AB.

Serum activin A and prostate specific antigen (PSA) determinations

Activin A and PSA serum concentrations were previously determined in PCA patients, BPH patients and in HS as described [27]. The reported detection limits were <78 pg/ml for activin A and <0.009 ng/ml for PSA respectively.

Statistical analysis

The D'Agostino–Pearson test was used to assess the normal distribution of the data. Therefore, statistical analysis was performed, where required, by the non parametric Mann–Whitney U test, the Kruskall–Wallis test, the Spearman rank correlation test or the parametric Student t test for independent samples. The diagnostic effectiveness of follistatin, activin A or PSA, alone or in combinations, in discriminating between M0 and M+ patients was determined by the receiver operating characteristic (ROC) curve analysis [28]. The significance of the difference between the areas under the ROC curves (AUCs) was assessed in accordance with Hanley and McNeil [29]. Data analysis was performed by using the Medcalc version 7.4.4 statistical software package (MEDCALC Mariakerke, Belgium). P values < 0.05 were considered statistically significant.

Fig. 1 Follistatin serum levels in healthy subjects (HS), in patients with benign prostate hyperplasia (BPH) and in patients with prostate cancer (PCa). *Horizontal bars* represent mean values. *P* values were calculated by the Mann–Whitney *U* test

Results

FLST serum levels were higher in PCa patients (mean 2.56 ± 1.5 ng/ml) than in BPH patients (mean 1.5 ± 0.72 ng/ml) or HS (mean 1.70 ± 0.45 ng/ml) or (Fig. 1). Conversely, BPH patients had FLST serum concentrations that were significantly lower than those measured in HS ($P = 0.025$) (Fig. 1). Interestingly, the ratio between the serum concentrations FLST and Act A (FLST/Act A ratio) was significantly higher in the HS group (mean 5.5 ± 3.3) than in M0 (mean 2.9 ± 1.4) or M+ (2.7 ± 1.2) or BPH patients (mean 2.1 ± 0.9), while no significant difference in this parameter was shown between M0 and M+ patients or between BPH and M0 or M+ patients (Fig. 2). In PCa patients, FLST levels were more elevated in M+ patients (median 2.7 ng/ml) as compared to M0 patients (median 1.75 ng/ml) (Table 1). Moreover, in cancer patients a positive correlation was observed between FLST and Act A or PSA serum concentrations (r = 0.59, $P = 0.0013$ and r = 0.56, $P = 0.024$, by the Spearman rank correlation test respectively) while no correlation was shown between serum FLST and Gleason score or extracapsular involvement or lymph node status (Table 1). Lastly, ROC curve generated
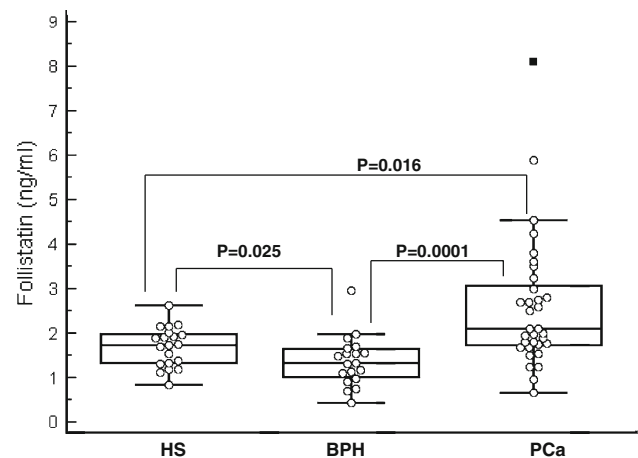
to assess the effectiveness of FLST in discriminating between M+ and M0 patients showed, in this respect, a good diagnostic performance for this molecule (Fig. 3). However, the AUC of FLST did not significantly differ from that of Act A or PSA (Fig. 3; Table 2). The combination of FLST with Act A or PSA, did not substantially resulted in an improved diagnostic accuracy as compared to that of each single molecule (Fig. 3; Table 2).

Discussion

Growing evidence suggests that the FLST/Act A system may be implicated in the pathogenesis of metastatic bone disease. This hypothesis is corroborated by several experimental observations which show that the balance between these molecules results deregulated in several pathological conditions associated with an altered bone turnover and in tumors which preferentially metastasize to the bone including prostate cancer [9–15, 17–26, 30–32]. In line with these observations, our data show that the serum levels of FLST are significantly increased in patients with prostate cancer and that the ratio between the serum concentrations of FLST and Act A (FLTS/Act A ratio) in these patients significantly differs from that measured in HS. Moreover, our studies highlight a positive correlation between circulating levels of FLST and the presence of bone metastasis or increased PSA levels. These findings further confirm and add to previous observations by Sardana et al. [26] showing a significant difference in FLST serum concentrations between subjects with or without prostate cancer. Our data are also consistent with those immunohistochemical and molecular biology studies which highlight a positive correlation between altered expression

Fig. 2 Serum FLST/Act A ratio in healthy subjects (HS), in patients with benign prostate hyperplasia (BPH) and in prostate cancer patients (PCa) with (M+) or without (M0) bone metastases. P values were calculated by the parametric Student t test for independent samples
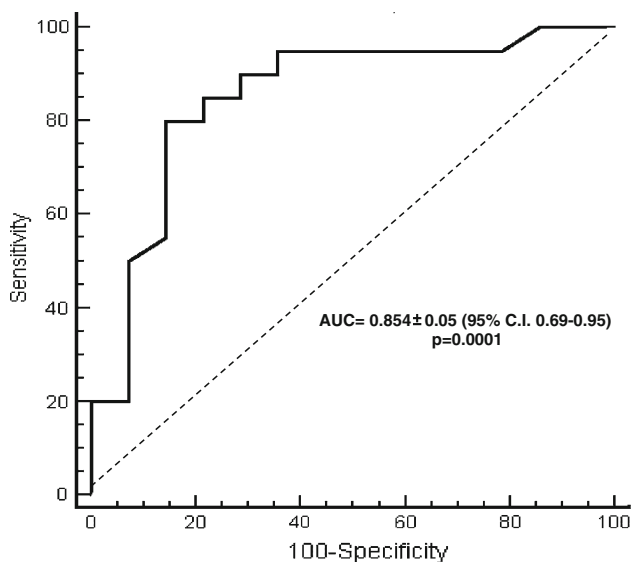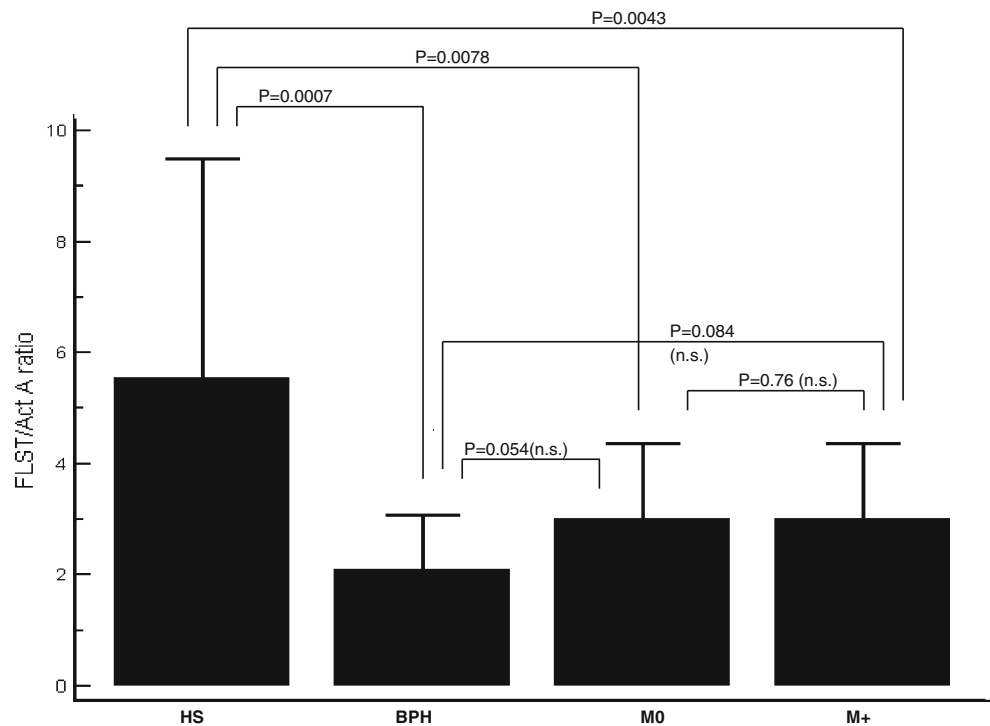

Fig. 3 Receiver operating characteristic (ROC) curve for follistatin to detect PCa patients with bone metastases. Detailed results of the ROC analysis are given in Table 2

level of FLST in prostate cancer and a more aggressive behaviour of this tumor [22–26, 31–33]. Although these observations reinforce the hypothesis of a possible involvement of FLST in PCa progression, the mechanisms by which this molecule may facilitate, in particular, bone metastasis formation in PCa patients remain to be unravelled. In this regard, some in vitro studies suggest that FLST may foster bone metastasis formation in prostate

cancer by modulating the activity of the bone morphogenic protein-7 (BMP-7), a member of the TGF-β family which is critical in the formation of osteoblastic lesions associated with prostate cancer metastases [34–37]. In addition, recent in vitro evidence suggests that FLST may indirectly facilitate bone metastasis formation by inhibiting activin type II receptor (ActRII) mediated signalling pathway [38]. This phenomenon appears to lead to the up-regulation of ADAM-15, a disintegrin which facilitate cell detachment by cleaving integrin molecules and whose overexpression appears to be strongly correlated with prostatic metastasis [39]. Intriguingly, this mechanism has been described as peculiar to prostate cancer and neuroblastoma [38]. Furthermore, experimental evidence suggests that this molecule may also facilitate the diffusion of malignant cells into the bone via the angiogenic route, through the activation of mechanisms which are independent from its Act A or BMPs inhibiting activity. In particular, some of these studies show that, FLST may interact with angiogenin, a 14-kDa protein which exerts its pro angiogenic functions by activating endothelial and smooth muscle cells and which promotes migration, invasion, proliferation and formation of tubular structure [40–43]. Additionally, it has been suggested that FLST, in concert with vascular endothelial growth factor (VEGF), may foster the formation of new blood vessels by stimulating the production of matrix-metalloproteinase-2 (MMP-2), a proteolytic enzyme which has been implicated in tumor angiogenesis and bone metastasis formation in prostate cancer [44–46]. On the

Table 2 Sensitivity, specificity and diagnostic accuracy of follistatin, alone or in combination with activin A and/or PSA, in the detection of patients with metastatic bone disease

Parameter	AUC ± SE[a] (95% C.I.)[b]	P value	Cut-off value[c] (ng/ml)	Sensitivity (%) (95% C.I.)[b]	Specificity (%) (95% C.I.)[b]	Accuracy (%)
Follistatin	0.846 ± 0.067 (0.68–0.95)	0.0001	1.99	80.0 (56.3–94.3)	84.6 (54.6–98.1)	81.8
Activin A	0.863 ± 0.062 (0.71–0.96)	0.0001	0.74	90.0 (68.3–98.8)	81.3 (54.6–98.3)	84.8
PSA	0.848 ± 0.068 (0.69–0.95)	0.0001	16.9	75.0 (50.9–91.3)	92.3 (64.0–99.8)	84.3
Follistatin + activin A	0.856 ± 0.065 (0.72–0.96)	0.0001	–	85.0 (62.1–96.8)	84.6 (51.6–98.1)	84.8
Follistain + PSA	0.787 ± 0.079 (0.61–0.91)	0.0003	–	65.0 (40.8–84.6)	92.3 (64.0–99.8)	75.7
Activin A + PSA	0.839 ± 0.068 (0.67–0.94)	0.0001	–	75.0 (50.9–91.3)	92.9 (66.1–99.8)	82.3
Follistatin + activin A + PSA	0.762 ± 0.083 (0.58–0.89)	0.016	–	60.0 (36.1–80.9)	92.3 (64.0–99.8)	72.7

[a] AUC = area under the curve ± standard error; [b] (95% C.I.) = 95% confidence interval; [c] cut-off values as determined by ROC curve analysis. No significant differences was observed between the AUCs (data not shown)

other hand, conflicting results have been generated from experimental in vivo studies regarding the promoting activity of FLST on tumor angiogenesis. In fact, Krneta et al. [47] recently showed that FLST may act as a potent angiogenesis stimulator in SCID mice transplanted with R30C human mammary tumor cells. Conversely, Ogino et al. [48] reported that, in NK cell-depleted SCID mice, FLST gene transfection resulted in the suppression of the experimental multiple organ metastases via inhibition of angiogenesis by small cell lung cancer (SCLC) cells. The discrepancies in these results are currently, in part, explained through the use of different animal tumor models and through the different role of the Act A-FLST system in the progression of different tumor types [47–51]. Therefore, further studies are needed to better assess the proangiogenetic effects of FLST on human tumors. Finally, ROC curves generated to asses the clinical value of circulating FLST as a gauge of bone metastasis in PCa patients, showed a good diagnostic performance of FLST in this respect. These data are consistent with recent findings from proteomic analysis studies which actually consider FLST as one of the novel candidate biomarkers of certain solid tumors including prostate cancer [25, 26, 49, 52, 53]. However, in our series of patients, the diagnostic performance of FLST did not result significantly superior to that of Act A or PSA. Although these data cast doubt on the specific role of serum FLST as a diagnostic gauge of bone metastasis in PCa patients, they indicate that this molecule may be of potential clinical interest as a circulating biomarker for the therapeutic management and follow-up of these patients when undergoing antimetastatic treatments. In conclusion, in line with other previous observations [47, 54], the present study suggests that, FLST may contribute to foster bone metastasis formation in PCa patients. In this scenario, FLST may be regarded as a new, potential molecular target in the treatment of metastatic bone disease while further investigations with a wider number of subjects is needed to better define the clinical role of this molecule as a biomarker that might be useful in the management of these patients.

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