PERGAMON

European Journal of Cancer 37 (2001) 486–491

European Journal of Cancer

www.ejconline.com

Prognostic importance of the soluble plasminogen activator receptor, suPAR, in plasma from rectal cancer patients

E. Fernebro [a,*], R.R. Madsen [b], M. Fernö [a], N. Brünner [b], P.-O. Bendahl [a], I.J. Christensen [b], A. Johnson [c], M. Nilbert [a]

[a] *The Department of Oncology, The Jubileum Institution, University Hospital, S-221 85 Lund, Sweden*
[b] *The Finsen Laboratory, Copenhagen University Hospital, Copenhagen, Denmark*
[c] *The Department of Oncology, Akademiska Hospital, Uppsala, Sweden*

Received 12 June 2000; received in revised form 16 October 2000; accepted 22 November 2000

Abstract

Colorectal cancer is one of the most common tumour types with approximately one third of the tumours located within the rectum. Rectal cancer differs somewhat from colon cancer, e.g. regarding the method of operation and the use of preoperative radiotherapy due to a tendency for local tumour recurrence. Proteolytic enzymes have been identified as key molecules in tumour invasion and metastasis, and factors within the urokinase-plasminogen activation (uPA) system have been associated with prognosis in several tumour types, including colorectal cancer. Recently, methods have been developed to analyse the soluble fraction of the plasminogen activator receptor (suPAR) in blood samples. An association between elevated suPAR levels and poor prognosis has recently been demonstrated in colorectal cancer. We have measured suPAR levels in pretreatment plasma samples from 173 rectal cancer patients in order to confirm its prognostic strength in this clinical entity. suPAR levels were determined in ethylenediamine tetraacetic acid (EDTA) plasma by a kinetic enzyme-linked immunosorbent assay (ELISA) and analysed with respect to sex, age, Dukes' stage, tumour differentiation grade and survival. In a univariate analysis, continuous suPAR plasma levels were associated with survival ($P < 0.001$) with shorter survival among patients with high suPAR values. Patients with suPAR values within the upper quartile had significantly shorter survival (hazard ratio (HR) 2.2, 95% confidence interval (CI) 1.3–43.7, $P = 0.002$). In a multivariate Cox analysis, increasing suPAR values predicted shorter survival independent from Dukes' stage and tumour differentiation grade with an adjusted HR of 2.2 per ng/ml suPAR (95% CI 1.2–4.0, $P = 0.01$). This study thus confirms that measurement of suPAR in preoperative plasma samples gives independent prognostic information in rectal cancer patients, higher values being associated with shorter survival.

Keywords: Soluble plasminogen activator receptor (suPAR); Rectal cancer; Prognostic marker

1. Introduction

Colorectal cancer is one of the most common cancer types and approximately one third of the carcinomas in the large intestine are situated in the rectum, which is defined as the bowel within 15 cm from the anus. The annual incidence of rectal cancer in Sweden is 25/100 000 in men and 15/100 000 in women. Local tumour recurrence, which is a major clinical problem, previously occurred in 20–50% of the patients [1], but after the introduction of a new surgical technique, total mesorectal excision (TME), the local recurrence rate has been reduced to about 5% [2]. Another means to reduce the local recurrence risk is addition of local preoperative radiotherapy [3]. Distant metastases develop in about half of the patients, most of whom will die from their cancer. Tumour stage (according to TNM or Dukes' classification) and histopathological tumour differentiation grade (low grade versus high grade) are widely used prognostic tools for colorectal cancer.

The aggressiveness of a tumour is reflected by its ability to invade adjacent tissue and to metastasise. Proteolytic enzymes produced by tumour cells and/or by supporting stromal cells, play a central role in this process [4,5]. The urokinase plasminogen activator

* Corresponding author. Tel.: +46-46177500; fax: +46-46147327.
E-mail address: eva.fernebro@onk.lu.se (E. Fernebro).

0959-8049/01/$ - see front matter
PII: S0959-8049(00)00422-6

(uPA) is a 52 kD serine protease, which is secreted as an inactive pro-enzyme (pro-uPA). uPA and pro-uPA bind to a specific cell surface receptor, uPAR, which consists of a three-domain 60 kD glycoprotein connected to the cell surface via a glycolipid anchor. uPA, free or receptor-bound, catalyses the conversion of plasminogen to plasmin, which plays an important role in the degradation of diverse substrates in the extracellular matrix [6]. Plasminogen activation by uPA is regulated by the inhibitors PAI-1 and PAI-2, which modulate the plasminogen activity by complex binding and internalisation through uPAR. In summary, the uPA/uPAR complex provides a means of directing the proteolytic activity to the cell surface and thereby influences the cell's invasive capacity [4–6]. High tumour tissue levels of uPAR have previously been associated with poor prognosis, e.g. in breast cancer [7,8] and in colorectal cancer [9]. The soluble fraction of uPAR, referred to as suPAR, is equivalent to the full-length three-domain receptor and cleavage products without the glycolipid cell surface anchor. Since uPAR is displayed on blood cells and is involved in platelet formation, senescence of monocytes or neutrophils, as well as platelet formation, may contribute to the normally measurable levels of suPAR in blood. Recently, a kinetic enzyme-linked immunosorbent assay (ELISA) was developed to measure suPAR in plasma [10]. suPAR in plasma from healthy blood donors showed no correlation with age, but the levels were somewhat higher in females than in males [10]. Elevated levels of circulating suPAR have been demonstrated in plasma from patients with, e.g. ovarian cancer, colorectal cancer and prostate cancer [10–12]. Recently, a significant correlation between increased levels of suPAR and shorter survival was demonstrated in preoperative blood samples from patients with colorectal cancer [13]. We have studied suPAR levels in plasma from 173 rectal cancer patients in order to validate the strength of suPAR as a prognostic marker.

2. Patients and methods

2.1. Patients

This study includes 173 patients (115 men (66%) and 58 women (34%)) from the southern Sweden healthcare region with histopathologically proven adenocarcinomas of the rectum. The median age was 66 years (range 31–91 years). Surgery was performed according to TME. Standard treatment also included preoperative radiotherapy to a total dose of 25 Gray (Gy) in five fractions [3]. Of the 173 patients, 134 (77%) with a primary resectable tumour received preoperative radiotherapy, 23 patients (13%) had a primary unresectable tumour and were therefore given prolonged radiotherapy of 50 Gy in 25 fractions, and 16 patients (9%) were not given any preoperative radiotherapy because of small tumours ($n=9$) or disseminated disease at the time of diagnosis ($n=7$). Blood samples were collected for suPAR analyses from consecutive patients between November 1994 and November 1998 and all samples were obtained before any treatment. Permission for the study was granted by the regional ethics committee. The tumours were classified according to Dukes' stage, which was A in 42 patients (24%), B in 54 (31%), C in 68 (39%) and D in 9 patients (5%) [14]. The tumours were classified according to histopathological tumour differentiation grade as high in 1 patient (1%), medium-high in 126 (73%) and poorly differentiated in 46 patients (27%). Tumours of high or medium-high differentiation are referred to as low-grade and tumours of poor differentiation as high-grade tumours. 3 patients died within 1 month of surgery (1 patient with a Dukes' D tumour from sepsis, 1 with a Dukes' A tumour from lung embolus and 1 patient with a Dukes' D tumour from cerebrovascular insult) and were excluded from the survival analysis. The median follow-up time was 3.4 years (range 1.3–5.5 years) for the survivors and 2.9 years (range 0.2–5.5 years) for all patients. During follow-up, 64 patients died, according to the death certificates in all cases from rectal cancer, but since the actual cause of death was not always verified, we used overall survival of all causes as the endpoint in the survival analysis.

2.2. Methods

Blood samples were drawn before any treatment and collected in ethylenediamine tetraacetic acid (EDTA)-containing anticoagulant tubes. Plasma was separated and frozen at −80°C until analysed. Immediately before performing the suPAR assay, the plasma samples were thawed rapidly at 37°C and diluted 1:10 in a sample dilution buffer of 50 mol/l phosphate, pH 7.2, 0.1 mol/l NaCl, 10 g/l bovine serum albumin (Fraction V, Boehringer Mannheim), and 1 g/l Tween 20. The plasma concentration of suPAR was determined by use of a modification of a kinetic ELISA as previously described [10]. In brief, this assay consists of using the R2 monoclonal antibody against human suPAR in the catching layer, a rabbit antibody against human suPAR in the detecting layer and a secondary monoclonal antirabbit immunoglobulin/alkaline phosphatase conjugate (Sigma Chemical Co., St Louis, MO, USA). Rate measurements of phosphatase enzyme activity were automatically collected over a 1 h period in a Ceres 900^{TM} plate-reader (BioTek Instruments, Winooski, VT, USA). KinetiCalc software (version 2.16; BioTek Instruments) was used to analyse the data and to calculate the rate of colour change for each well by linear regression analysis. The suPAR concentration of each

plasma sample was calculated by use of a four-parameter fitted standard curve computed from the rates for the recombinant suPAR standards. The detection limit of the assay was 3 pg/ml, the interassay variation of a citrate plasma pool ($n=6$) was 12% and the intra-assay variation was 6.7%. Samples from all patients were analysed in duplicate.

2.3. Statistical analysis

The software package Stata 6.0 was used for the statistical analyses [15]. Differences in the median suPAR levels in subgroups of quantative factors were evaluated using the Kruskal–Wallis test and boxplots. The median and the upper and lower quartiles define the box whereas the whiskers extend to the upper and lower adjacant values. The upper adjacent value is defined as the largest data point less than or equal to 1.5× the Inter Quartile Range (IQR) and the lower adjacent value as the smallest data point greater than or equal to 1.5×IQR. Data points more extreme than the adjacent values are plotted individually. The width of each box is proportional to the corresponding number of observations. Spearmans' rho was used as a measure of correlation between suPAR and age. Associations between suPAR, categorised by the median, and other factors were evaluated using χ^2-tests. Overall survival was analysed using Kaplan–Meier estimates, logrank tests and Cox regression analysis. The Kaplan–Meier curves were curtailed when no more than five individuals remained in the risk sets. Proportional hazards assumptions were checked using Schoenfeld's test. None of the factors included in the multivariate analysis was found to violate the assumption. All tests were two-sided and the significance level was set at 5%.

3. Results

All 173 plasma samples contained measurable levels of suPAR with a median value of 1.17 ng/ml (range 0.48–2.74 ng/ml). The suPAR levels showed a weak correlation with age (Spearman's rho 0.31) and the median suPAR values were also higher in females (1.2 ng/ml) than in males (1.1 ng/ml) ($P=0.04$). The median suPAR values were about the same in Dukes' stage A–C tumours, whereas the median suPAR value was significantly higher in Dukes' stage D tumours ($P=0.01$, two-group comparison Dukes' stages A–C versus D) (Fig. 1). suPAR values above the median were found in 48% ($n=79$) of the 164 patients with Dukes' stages A–C tumours, but in 7/9 patients (78%) with Dukes' D tumours. The median suPAR values were also significantly higher in high-grade tumours compared with low-grade tumours ($P=0.04$) (Fig. 1). Among low-grade tumours 45% ($n=57$) had suPAR levels above the median compared with 63% ($n=29$) of the high-grade tumours.

3.1. Univariate analysis

The 3 patients who died from postoperative complications were excluded from the survival analyses, thus analyses are based on the remaining 170 patients. Survival analysis using the Kaplan–Meier method showed that patients with suPAR values above the median (1.17 ng/ml) had a significantly shorter survival than patients with values below the median (hazard ratio (HR) 1.8, 95% confidence interval (CI) 1.1–2.9, $P=0.02$). When the patients were divided into four groups using the quartile values for plasma suPAR, a significant difference was seen in survival between the groups ($P=0.01$, four-group comparison). No significant difference in survival was found between the three quartiles with lower suPAR values ($P=0.63$). In contrast, patients with values within the upper quartile had a significantly shorter survival compared with the lower three quartiles (HR 2.2, 95% CI 1.3–43.7, $P=0.002$) (Fig. 2). The 3-year survival for the quartile with the highest suPAR values was 52% (95% CI 35–66%) compared with 73% (95% CI 64–80%) for patients with suPAR values in the lower three quartiles. When analysed univariately as a continuous variable on a linear scale, suPAR was also found to correlate with survival with an estimated HR of 3.0 (95% CI 1.6–5.6, $P<0.001$) per ng/ml suPAR. As expected, Dukes' stage and tumour differentiation grade were significantly associated with survival, whereas sex and age were not. The 3-year survival according to Dukes' stage was 91% (95% CI: 74–97%) for patients with Dukes' A tumours, 75% (95% CI: 59–85%) for

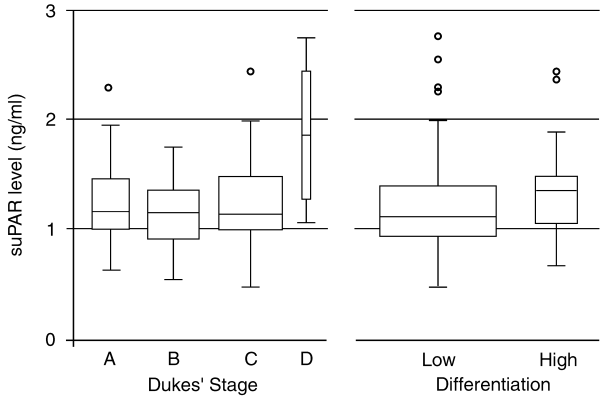
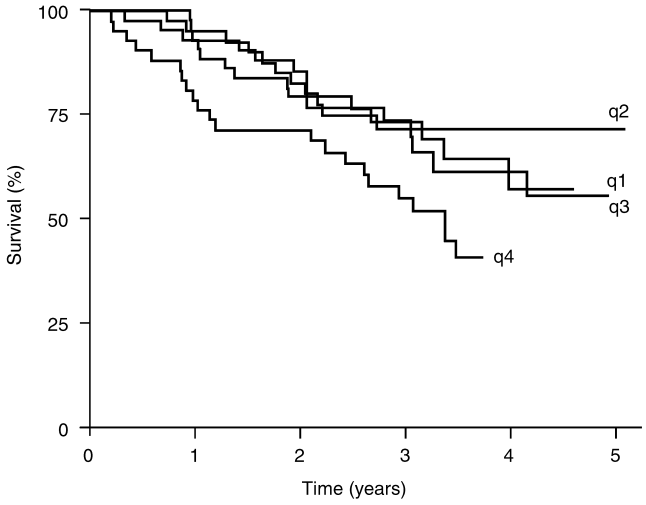


Fig. 1. Boxplots demonstrating suPAR levels in relation to Dukes' stage and histopathological tumour differentiation grade. The left panel shows the median suPAR values for Dukes' stage A–C tumours, compared with the small group of patients with Dukes' stage D tumours ($P=0.01$, two-group comparison Dukes' stage A–C versus D). The right panel demonstrates suPAR values in low-grade tumours compared with high-grade tumours ($P=0.04$). The elements of the boxplots are defined in the statistical analysis section. suPAR, soluble fraction of the urokinase plasminogen activator receptor.



Patients at risk								Deaths
q1	<1.00	44	41	34	18	8	4	14
q2	1.00 – 1.17	41	39	33	22	15	6	11
q3	1.18 – 1.45	43	40	30	20	11	3	15
q4	>1.45	42	33	29	18	4	0	24

Fig. 2. Kaplan–Meier curve demonstrating overall survival according to quartile (q) suPAR values.

Dukes' B, 54% (95% CI: 41–66%) for Dukes' C and 25% (95% CI: 4–56%) for Dukes' D. The corresponding 3-year survival according to tumour differentiation was 77% (95% CI: 69–84%) for patients with low-grade tumours and 43% (95% CI: 28–58%) for those with high-grade tumours. However, no statistically significant discrimination between high and low suPAR levels regarding survival could be obtained in Dukes' B or Dukes' C stage patients.

3.2. Multivariate analysis

In a multivariate Cox analysis including the factors sex, age, Dukes' stage, tumour differentiation grade and suPAR, significant independent prognostic information was obtained from Dukes' stage, differentiation grade and suPAR values, but not from sex and age (Table 1). suPAR analysed as a continuous variable in the Cox analysis gave prognostic information independent of Dukes' stage and differentiation grade with an adjusted HR of 2.2 (95% CI 1.2–4.0, $P=0.01$) (Table 1). In addition, suPAR categorised into quartiles gave significant prognostic information independent from Dukes' stage and differentiation grade (HR 1.9, 95% CI 1.1–3.1, $P=0.02$).

4. Discussion

Studies of uPAR levels in tumour tissue extracts, which have demonstrated that high tissue levels are related to a worse prognosis in several tumour types, together with the identification of suPAR as a novel serological prognostic marker in, e.g. ovarian cancer, colorectal cancer and prostate cancer, confirm the importance of the uPAR system in cancer [10–12]. uPAR-expression studies in colorectal cancer have shown an increased immunoreactivity on the surface of tumour cells and tumour infiltrating macrophages [16]. The biological role of suPAR is unclear, but the increased blood suPAR levels in cancer patients are probably caused by release from tumour cells or stromal cells in the cancerous tissue and experimental data support a relationship between suPAR level and tumour burden [17]. *In vitro* experiments have suggested that increased amounts of circulating suPAR have biological importance; suPAR can induce chemotaxis, acts as a scavenger for uPA and thereby inhibits cell proliferation, and through interaction/binding with vitronectin suPAR/uPA complexes may influence cell migration [18–20].

Table 1
Multivariate Cox analysis including Dukes' stage, tumour differentiation grade and suPAR plasma level in 170 rectal cancer patients

Factor	Hazard ratio (95% CI)	*P* value
Dukes' stage		
B versus A	2.9 (1.0–8.8)	0.06
C versus A	5.3 (1.9–15)	0.002
D versus A	17 (4.6–60)	< 0.001
Tumour differentiation grade (high versus low)	2.8 (1.6–4.9)	< 0.001
suPAR (continuous variable)	2.2 (1.2–4.0)	0.01

95% CI, 95% confidence interval suPAR, see Fig. 1.

In this retrospective study, suPAR values in blood samples from rectal cancer patients were found to be associated with survival. The risk increase was 3.0 per ng/ml suPAR (95% CI 1.6–5.6) with shorter survival among patients with high suPAR values ($P < 0.001$). In the multivariate analysis, including the factors sex, age, Dukes' stage and tumour differentiation grade and suPAR, independent prognostic information was obtained using the upper quartile for the suPAR values and also when analysing suPAR as a continuous variable. Furthermore, patients with suPAR levels within the upper quartile had a significantly shorter survival than patients with values in the lower three quartiles (HR 2.2, 95% CI 1.3–43.7, $P = 0.002$) and the 3-year survival for the two groups was 52% (95% CI 35–66%) and 73% (95% CI 64–80%), respectively (Fig. 2). When analysing the importance of suPAR relative to survival, we found that our data supported a non-linear association between suPAR and tumour-related death; suPAR levels below the upper quartile seemed to be comparable from a prognostic point of view, whereas suPAR values within the upper quartile reflected a worse prognosis. Notably, among the 7 patients with the highest absolute suPAR values, 6 (all of whom had Dukes' stage C or D tumours) died from their tumour within 15 months of diagnosis. Furthermore, among the 9 patients with Dukes' D tumours, the 2 patients with the lowest suPAR values are alive 4 and 5 years after diagnosis, respectively.

This study confirms the results from an earlier and the first study on the prognostic value of suPAR measurements in preoperative blood samples from patients with colorectal cancer. In a study including 591 patients with colorectal cancer, Stephens and colleagues demonstrated an increased risk of mortality for patients with suPAR values above the median [13]. Our study confirms the observation that high suPAR values were significantly associated with poor survival and demonstrates, in an independent set of patients, that the prognostic information of suPAR is applicable also for patients with rectal cancer. Clinically, the development of prognostic markers which can be used before treatment would be valuable in order to determine which patients would benefit from adjuvant therapy, e.g. addition of radiotherapy and/or chemotherapy. The suPAR values could in the study by Stephens and colleagues discriminate between high-risk and low-risk patients with Dukes' stage B and stage C tumours [13]. We could not, however, based on suPAR levels, predict which patients with Dukes' stages B or C tumours were at high or low risk for tumour recurrence. This discrepancy may be explained by the larger patient material and a longer follow-up time in the former study, but might also reflect differences in treatment since the TME method for operation, combined with preoperative radiotherapy in the majority of patients, has been introduced in the current study. This optimised treatment may thus have influenced survival among the patients with Dukes' stages B and C tumours with a suggested poor prognosis due to high suPAR values. We conclude that evaluation of factors within the plasminogen activation system, and measurements of suPAR in preoperative blood samples in particular, may be clinically valuable prognostic markers in rectal cancer.

Acknowledgements

This study was financially supported by the GAE Nilsson Cancer Foundation, the Mrs B Kamprad Foundation, the M Bergqvist Foundation and the Gustaf V Jubilee Fund. We would like to acknowledge Dr Anders Johnsson for help with collecting patient data, Kerstin Svensson and Karin Nilsson for help with collecting the blood samples, and Eva Långström and Eva Rambech for helpful technical assistance.

References

1. McArdle CS, Hole D. Impact of variability among surgeons on postoperative morbidity and mortality and ultimate survival. *Br Med J* 1991, **302**, 1501–1505.
2. MacFarlane JK, Ryall RD, Heald RJ. Mesorectal excision for rectal cancer. *Lancet* 1993, **341**, 457–460.
3. Swedish Rectal Cancer Trial. Improved survival with preoperative radiotherapy in resectable rectal cancer. *New Engl J Med* 1997, **336**, 980–987.
4. Duffy MJ. Proteases as prognostic markers in cancer. *Clin Cancer Res* 1996, **2**, 613–618.
5. Andreasen PA, Kjöller L, Christensen L, Duffy MJ. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int J Cancer* 1997, **72**, 1–22.
6. Brünner N, Nielsen HJ, Hamers M, Christensen IJ, Thorlacius-Ussing O, Stephens RW. The urokinase plasminogen activator receptor in blood from healthy individuals and patients with cancer. *APMIS* 1999, **107**, 160–167.
7. Duggan C, Maguire T, McDermott E, O'Higgins N, Fennelly JJ, Duffy MJ. Urokinase plasminogen activator and urokinase plasminogen activator receptor in breast cancer. *Int J Cancer* 1995, **61**, 597–600.
8. Gröndahl-Hansen J, Peters HA, van Putten WLJ, *et al.* Prognostic significance of the receptor for urokinase plasminogen activator in breast cancer. *Clin Cancer Res* 1995, **1**, 1079–1087.
9. Ganesh S, Sier CF, Heerding MM, Griffioen G, Lamers CB, Verspaget HW. Urokinase receptor and colorectal cancer survival. *Lancet* 1994, **344**, 401–402.
10. Stephens RW, Pedersen AN, Nielsen HJ, *et al.* ELISA determination of soluble urokinase receptor in blood from healthy donors and cancer patients. *Clin Chem* 1997, **43**, 1868–1876.
11. Sier CFM, Stephens R, Bizik J, *et al.* The level of urokinase-type plasminogen activator receptor is increased in serum of ovarian cancer patients. *Cancer Res* 1998, **58**, 1843–1849.
12. McCabe NP, Angwafo FF, Zaher A, Selman SH, Kouinche A, Jankun J. Expression of soluble urokinase plasminogen activator receptor may be related to outcome in prostate cancer patients. *Oncol Rep* 2000, **4**, 879–882.
13. Stephens RW, Nielsen HJ, Christensen IJ, *et al.* Plasma urokinase receptor levels in patients with colorectal cancer: relationship to prognosis. *J Natl Cancer Inst* 1999, **91**, 869–874.

14. American Joint Committee on Cancer. *Cancer Staging Manual*. 5th edn. Philadelphia, Lippincott-Raven, 1998.
15. StataCorp. *Stata Statistical Software,* release 6.0. College Station, TX, Stata Corporation, 1999.
16. Pyke C, Kristensen P, Ralfkiaer E, *et al*. Urokinas-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. *Am J Pathol* 1991, **138**, 1059–1067.
17. Holst-Hansen C, Hamers MJ, Johannessen BE, Brünner N, Stephens RW. Soluble urokinase receptor released from human carcinoma cells: a plasma parameter for xenograft tumor studies. *Br J Cancer* 1999, **81**, 203–211.
18. Resnati M, Guttinger M, Valcamonica S, Sidenius N, Blasi F, Fazioli F. Proteolytic cleavage of the urokinase receptor substitutes for the agonist-induced chemotactic effect. *EMBO J* 1996, **15**, 1572–1582.
19. Höyer-Hansen G, Behrendt N, Ploug M, Danö K, Preissner KT. The intact urokinase receptor is required for efficient vitronectin binding: receptor cleavage prevents ligand interaction. *FEBS Lett* 1997, **420**, 79–85.
20. Wilhelm O, Weidle U, Höhl S, Rettenberger P, Schmitt M, Graeff H. Recombinant soluble urokinase receptor as a scavenger for urokinase-type plasminogen activator (uPA). Inhibition of proliferation and invasion of human ovarian cancer cells. *FEBS Lett* 1994, **337**, 131–134.