Plasma Urokinase Receptor Levels in Patients With Colorectal Cancer: Relationship to Prognosis

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Background: The proteolytic enzyme plasmin, which is generated from the precursor plasminogen by the action of urokinase plasminogen activator, is thought to play a role in tumor cell invasion and metastasis. Urokinase plasminogen activator receptor (uPAR) is functionally involved in the cell surface activation (i.e., cleavage) of plasminogen. Increased tumor tissue levels of uPAR are associated with poor prognosis in several types of cancer. This retrospective study was undertaken to test the relationship between preoperative plasma levels of soluble uPAR (suPAR) and survival in patients with colorectal cancer. *Methods:* suPAR levels in preoperative plasma from 591 patients with colorectal cancer were determined by use of a kinetic enzyme-linked immunosorbent assay and analyzed with respect to associations with postoperative survival, Dukes' stage, age, and serum carcinoembryonic antigen level. Plasma suPAR measurements were log transformed for survival analysis, which employed the Kaplan–Meier method and the Cox proportional hazards model. All P values reported are two-sided. *Results:* Univariate analysis, using the log-transformed suPAR concentrations, demonstrated that there was an increasing risk of mortality with increasing plasma suPAR level ($P<.0001$). An arbitrary cut point, the median for all patients (1.37 ng/mL), divided patients with Dukes' stage B, C, or D disease into statistically different prognostic groups. In multivariate Cox analysis including Dukes' stage, age, and carcinoembryonic antigen level, the suPAR concentration independently predicted survival ($P<.0001$). *Conclusions:* The preoperative plasma suPAR level independently predicted survival of patients with colorectal cancer. Further studies of plasma suPAR in patients with cancer are needed to evaluate the utility of plasma suPAR measurements and cut points in identifying high-risk patients among those with early stage disease. [J Natl Cancer Inst 1999;91:869–74]

In an effort to more selectively use adjuvant treatment for patients with cancer, many investigators have searched for prognostic markers. Successful identification and selection of high-risk patients for adjuvant treatment could spare a substantial number of low-risk patients from the side effects of chemotherapy. Moreover, the exclusion of low-risk patients could potentially improve the efficiency of adjuvant treatment studies, since it would reduce both the number of patients needed and the follow-up time required for valid assessment.

It is now well established that proteolytic enzymes produced by cancer cells and/or cells in the tumor stroma are involved in the intensive tissue remodeling that accompanies cancer cell invasion and metastasis *(1–3).* Among several enzyme systems expressed in cancer tissue, plasmin generated by urokinase plasminogen activator (uPA) is thought to play a key role in tissue degradation *(4,5),* activation of pro-metalloproteases *(6),* activation of cytokines *(7),* and angiogenesis *(8),* all of which could lead to an increase in the metastatic potential of the cancer cells. uPA is secreted as an inactive proenzyme *(9),* which localizes on cell surfaces *(10)* by binding through its epidermal growth factor-like domain to a specific high-affinity cell surface receptor (urokinase plasminogen activator receptor [uPAR]) *(11).* uPAR is a cell surface glycoprotein with a molecular mass of 55–60 kd and consists of three homologous protein domains, all of which are required for high-affinity binding of uPA. At the carboxyl terminal of domain 3, uPAR is anchored to the cell membrane by a glycosylphosphatidylinositol moiety *(11).* Compared with activation of uPA proenzyme in free solution, activation of uPAR-bound proenzyme is strongly enhanced as a result of the proximity of cell surface-bound plasminogen and plasmin *(12–14).* There is considerable experimental evidence that uPAR is functionally involved in cancer invasion *(15–18),* consistent with its ability to concentrate and enhance uPA activity on cell surfaces *(10).*

We and others have previously reported the association of uPAR levels in tumor tissues with prognosis for patients with squamous cell lung cancer *(19),* colon cancer *(20),* and breast cancer *(21).* In the latter study, it was found that a fraction representing soluble uPAR (suPAR), i.e., uPAR protein without the glycolipid anchor, was inversely related to survival. We have previously found that suPAR is normally present at low levels in the blood *(22)* but that the levels are increased in patients with non-small-cell lung cancer *(23),* metastatic breast cancer *(24),* Dukes' stage D colorectal cancer *(24),* and ovarian cancer *(25),* probably as a result of the release of suPAR into the circulation from tumors.

We have now undertaken a retrospective study of suPAR levels in preoperative plasma from 591 patients who had surgery for colorectal cancer. We tested for an association between the levels of suPAR and patient survival.

SUBJECTS AND METHODS

Patients. This study included 591 patients who underwent surgery for colorectal cancer. Blood samples were obtained from all patients before surgery. Written informed consent was also obtained from all patients before surgery in accordance with the Helsinki declaration, and permission was granted by the local ethical committees of the Hvidovre Hospital and the Aalborg Hospital in Denmark. All patients had histologically verified adenocarcinoma of the colon or rectum. Fifty-nine patients (10%) were classified as having Dukes' stage A disease, 219 (37%) as having Dukes' stage B disease, 170 (29%) as having Dukes' stage C disease, and 143 (24%) as having Dukes' stage D disease *(26).*

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Patients with Dukes' stage A, B, or C disease underwent complete resection of their tumors, whereas patients with Dukes' stage D disease had resection of their primary tumor and distant metastases whenever possible. None of the patients received adjuvant chemotherapy. Complete clinical data, including age, sex, Dukes' stage, serum carcinoembryonic antigen (CEA) level, and overall survival after the surgery were registered for all patients. The median age of patients at surgery was 69 years (range, 33–90 years), and there were 354 males and 237 females. The median follow-up time was 5.1 years (range, 4.0–6.3 years). During the observation period, 333 patients (56%) died. Seventeen deaths that occurred within 1 month of surgery from postoperative complications were censored. Recording of survival for all patients surviving 1 month or more was based on death from all causes.

Sampling of blood. Blood samples (5 mL) for suPAR analysis were taken preoperatively from patients on the day of their surgery and were collected in ethylenediamine tetraacetate (EDTA)-containing anticoagulant tubes (Becton Dickinson, Mountain View, CA). Plasma was separated within 1.5 hours and stored frozen at −80 °C until analyzed. Immediately before suPAR assay, the plasma samples were thawed rapidly at 37 °C and diluted 1 : 10 as previously described (24). Blood (5 mL) was also collected preoperatively for serum CEA measurement, by use of the Immulite™ CEA assay kit (Diagnostics Products Corporation, Los Angeles, CA).

suPAR analysis. The plasma concentration of suPAR was determined by use of a modification of a new kinetic enzyme-linked immunosorbent assay (ELISA) that meets strict criteria of specificity and sensitivity (24). Briefly, the ELISA consisted of a catching layer of the monoclonal antibody R2 and a detection layer of rabbit polyclonal antibodies to human uPAR, i.e., an inversion of the two layers in the described ELISA (24). This modification eliminated a background signal found in approximately 4% of individuals, independent of cancer diagnosis. The R2 monoclonal antibody has high affinity for domain 3 of the human uPAR molecule, so that both the full-length (domains 1 + 2 + 3) and proteolytically cleaved (domains 2 + 3) forms of suPAR (11) were measured by this assay. A monoclonal anti-rabbit immunoglobulin/alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) was used in the final step, so that rate measurements for phosphatase enzyme activity could be automatically collected over a 1-hour incubation period in a Ceres 900™ plate reader (Bio-Tek Instruments, Winooski, VT) (24). KinetiCalc software (version 2.16; Bio-Tek Instruments) was used to manage the data and to calculate the rate of color 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 absolute concentration of the recombinant suPAR standard was previously determined by amino acid analysis (27). The limit of detection for the assay was 3 pg/mL or approximately 0.3% of the median concentration found in donor plasma. The intra-assay variation for a plasma pool was 4.8% (n = 21), and the inter-assay variation for 30 successive assays of aliquots of the same plasma pool (on different days) was 7.6%. suPAR was evidently stable in frozen plasma for at least several months. When recombinant suPAR was added to plasma as an internal control, 97% of the standard could be detected by ELISA. Specificity was rigorously controlled by plasma immunoabsorption experiments as described previously (24).

Statistical analyses. The SAS® software package (version 6.12; SAS Institute, Cary, NC) was used to manage the patient data and to perform all statistical analyses. The plasma suPAR measurements were log transformed (i.e., ln suPAR) for survival analysis; for graphical representation, the patients were stratified into four groups based on the suPAR value, such that each stratum yielded an equal number of events (deaths of patients). For each Dukes' stage, the median value of plasma suPAR determined for all patients was used for arbitrary dichotomization. The Kaplan–Meier method was used to estimate survival probabilities, and the logrank test was used to test for equality of strata. The Cox proportional hazards model was used for analysis of continuous covariates as well as for multivariate analysis. The assumption of proportional hazards was verified graphically. Rank statistics were used to calculate correlation coefficients and to test hypotheses on location. Tests of independence were done with the use of the chi-squared test. The significance level was set to 5%. The expected survival for patients in each Dukes' stage was calculated for age- and sex-matched cohorts (28) by use of official vital statistics recorded and tabulated for the Danish population (29). All P values reported are two-sided.

RESULTS

Levels of suPAR in Plasma

suPAR was measured by a modified kinetic ELISA method in EDTA-anticoagulated plasma obtained preoperatively from each patient with colorectal cancer. All the plasma samples had measurable levels of suPAR, with a median value of 1.37 ng/mL (range, 0.46–8.0 ng/mL). When the patient data were broken down according to Dukes' stage, there were statistically significant differences in plasma suPAR levels, with Dukes' stage A being the lowest and Dukes' stage D being the highest (Kruskal–Wallis test, P = .001). Nevertheless, it was clear that higher levels of plasma suPAR were not restricted to advanced disease. The means, standard deviations, medians, and interquartile ranges for plasma suPAR are summarized in Table 1. There was a significant but relatively weak correlation between the suPAR levels and age of patients with cancer (Spearman's rho = .28; P<.0001), but no significant association was found between sex and level of suPAR (Wilcoxon rank sum test, P = .11). The median CEA level was 3.8 ng/mL (range, 0.34–9800 ng/mL), and there was a significant but relatively weak correlation between the level of CEA and the level of suPAR (Spearman's rho = 0.31; P<.0001).

Prognostic Value of Preoperative Plasma suPAR Level

Treated as a continuous variable, the log-transformed plasma level of suPAR (i.e., ln suPAR) was statistically significantly associated with survival (Cox regression model, P<.0001; Table 2); higher levels of suPAR were found in plasma from patients who had a shorter survival. For survival analysis in which the Kaplan–Meier method was used, the patients were divided into four strata based on the plasma suPAR value, such

Table 1. Summary of plasma suPAR* levels for patients with colorectal cancer

Dukes' stage	Mean suPAR ± SD,† ng/mL	Median, ng/mL	Interquartile range Centile‡	Interquartile range ng/mL
A	1.30 ± 0.57	1.20	0%–25%	0.63–0.96
			>25%–50%	>0.96–1.20
			>50%–75%	>1.20–1.50
			>75%–100%	>1.50–3.89
B	1.49 ± 0.63	1.35	0%–25%	0.58–1.12
			>25%–50%	>1.12–1.35
			>50%–75%	>1.35–1.72
			>75%–100%	>1.72–5.99
C	1.33 ± 0.45	1.31	0%–25%	0.46–0.99
			>25%–50%	>0.99–1.31
			>50%–75%	>1.31–1.60
			>75%–100%	>1.60–2.75
D	1.84 ± 0.95	1.69	0%–25%	0.50–1.20
			>25%–50%	>1.20–1.69
			>50%–75%	>1.69–2.20
			>75%–100%	>2.20–8.00

*suPAR = soluble urokinase plasminogen activator receptor.

†SD = standard deviation.

‡Centile from percentile distribution of plasma suPAR levels for all patients.

Table 2. Summary of survival analyses of patients with colorectal cancer

Parameter	Univariate analysis (n = 591)		Multivariate analysis (n = 580)*	
	P, two-sided	HR (95% CI)†	P, two-sided	HR (95% CI)†
Dukes' stage				
B vs. A	<.0001	2.4 (1.2–4.6)	.04	2.0 (1.0–3.9)
C vs. A	<.0001	4.7 (2.5–9.1)	<.0001	4.4 (2.3–8.5)
D vs. A	<.0001	19.3 (10.1–37.0)	<.0001	15.0 (7.8–29.2)
Sex				
Female vs. male	.48	0.9 (0.7–1.2)	.19	0.9 (0.7–1.1)
Age in years	.005	1.3‡ (1.1–1.7)	.02	1.3‡ (1.1–1.7)
Serum carcinoembryonic antigen (median)	<.0001	2.0 (1.6–2.5)	.18	1.1 (0.9–1.5)
suPAR (ln suPAR)§	<.0001	2.8‖ (2.2–3.7)	<.0001	1.9‖ (1.4–2.5)

*Eleven missing serum carcinoembryonic antigen data.

†HR = hazard ratio; 95% CI = 95% confidence interval. suPAR = soluble urokinase plasminogen activator receptor.

‡HR for two patients differing in age by 20 years.

§suPAR = soluble urokinase plasminogen activator receptor; ln suPAR = log-transformed suPAR.

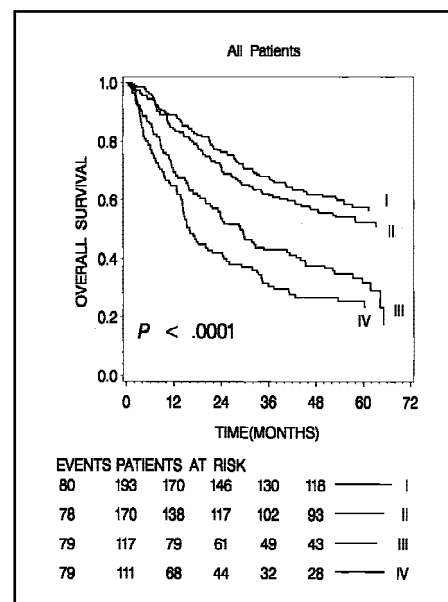‖HR for two patients differing in ln suPAR by 1.0 (i.e., differing in plasma suPAR levels by a factor of 2.7).


Fig. 1. Level of soluble urokinase plasminogen activator receptor (suPAR) in preoperatively sampled plasma and overall survival of 591 patients with colorectal cancer. Patients were divided into four strata based on the plasma suPAR values, yielding closely similar numbers (78–80) of events (deaths of patients) in each stratum. Thus, the four curves in the figure represent patients with plasma suPAR <1.16 ng/mL (I), 1.16–1.52 ng/mL (II), 1.53–1.95 ng/mL (III), and >1.95 ng/mL (IV). The P value (two-sided) was calculated by use of the logrank test, and the hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated by use of the Cox regression model. The numbers of patients at risk after each 12-month interval up to 48 months are indicated below the figure. Patients in stratum II had an HR of 1.2 (95% CI = 0.9–1.6; P = .28) compared with patients in stratum I; patients in stratum III had an HR of 2.2 (95% CI = 1.6–3.0; P = .0001) compared with patients in stratum I, and patients in stratum IV had an HR of 2.9 (95% CI = 2.1–3.9; P = .0001) compared with patients in stratum I. The survival probabilities at 24 months and 48 months (plus 95% CIs) are 76% (70–82) and 61% (54–68), respectively, for stratum I and 41% (32–50) and 27% (18–35), respectively, for stratum IV.

that each stratum yielded an equal number of events (deaths of patients; *see* Fig. 1). This procedure produced strata with different hazard ratios (HRs) (*see* legend to Fig. 1), and the analysis indicated a continuously increasing risk of mortality with increasing suPAR level.

We next tested the prognostic significance of suPAR level in each of the four Dukes' stages (Fig. 2). For this purpose, we used the simplest test in which the patients were divided by an arbitrary cut point, and we chose for the cut point the median plasma suPAR level for all patients (1.37 ng/mL). *Note* that the results for patients with Dukes' stage A disease shown in Fig. 2 represent only 10 events among the 59 patients with Dukes' stage A disease over the observation period, consistent with the good prognosis expected for patients with tumors detected at a very early stage that were clearly localized and amenable to complete surgical removal. In Dukes' stages B, C, and D, application of the median plasma suPAR level for all patients resulted in each case in division of patients into prognostic groups with significantly different survival (Fig. 2). Higher suPAR levels in each of these Dukes' stages were associated with significantly shorter survival.

It was of some clinical interest that, in the group with Dukes' stage B disease, representing relatively early stage and potentially surgically curable disease, patients with plasma suPAR levels above the median (106 of 219 patients, 48%) had an HR of 2.8 (95% confidence interval [CI] = 1.8–4.6; P<.0001) compared with those with levels below the median.

Since recording of overall survival in this study was based on deaths from all causes, we calculated the expected survival for a cohort drawn from the general Danish population and matched with the patients with Dukes' stage B disease with respect to age and sex (29). As shown in Fig. 2, patients with Dukes' stage B disease who had plasma levels of suPAR below the median (113 of 219 patients, 52%) had a death rate indistinguishable from that expected in the age- and sex-matched cohort. Patients with Dukes' stage B disease who had higher plasma suPAR levels had shorter survival (testing the hypothesis that the number of events is equal to the expected number by a chi-squared test, P<.0001). We would caution, however, that the median for all patients was chosen as an arbitrary cut point in testing the suPAR level for its relationship to prognosis, and it is not implied that this is a clinically useful cut point for patients with Dukes' stage B disease; testing of cut points for clinical applications is beyond the scope of this study.

Patients with Dukes' stage C disease who had plasma suPAR levels above the median level (72 of 170 patients, 42%) had an HR of 1.8 (95% CI = 1.2–2.6; P = .005) relative to those who had levels below the median level (Fig. 2). Thus, the median plasma suPAR level could also divide patients with more advanced, less surgically curable disease into groups with differing survival. However, virtually all patients with Dukes' stage C disease had a significantly higher death rate than an age- and sex-matched cohort drawn from the general population.

Division by the median in patients with Dukes' stage D disease also showed a relationship between suPAR level and prognosis (Fig. 2), but all patients with this disease stage had relatively poor survival due to disseminated disease. In summary, the results that we obtained using an arbitrary cut point for analyses of prognosis in each Dukes' stage show that the suPAR level is a statistically significant prognostic factor in Dukes' stages B, C, and D, but its greatest potential value is as a clinical marker in early stage disease.

Multivariate Analysis

Multivariate Cox analysis of the survival data was performed and included the clinical parameters Dukes' stage, sex,
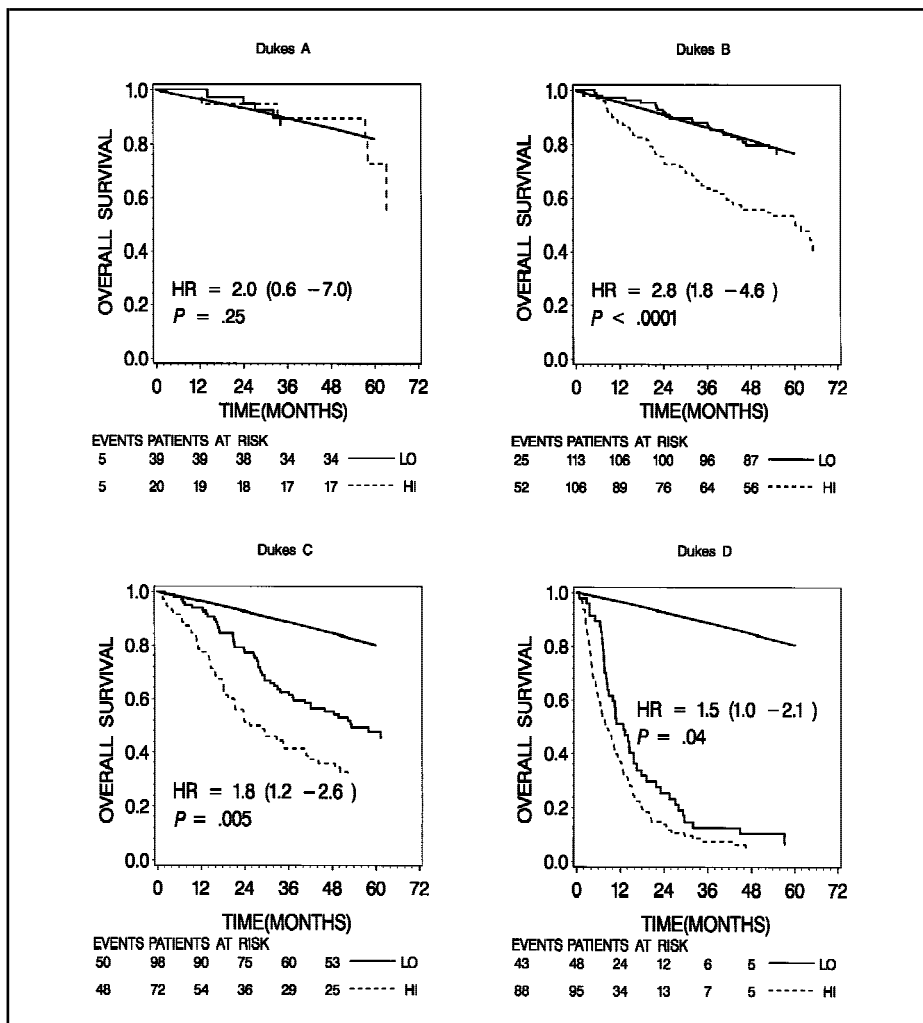
Fig. 2. Preoperative plasma suPAR level and overall survival for patients with colorectal cancer in each of the four Dukes' stages. Patients in each stage were divided into two groups consisting of those above (**stepped solid curve**) and those below (**stepped dashed curve**) an arbitrary cut point, for which we chose the median plasma suPAR level determined for all patients (1.37 ng/mL). The survival curve for a Dukes' stage-specific, age- and sex-matched cohort drawn from the general Danish population is also plotted (**continuous solid line**) for each Dukes' stage. The *P* value (two-sided) was calculated by use of the logrank test, and hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated by use of the Cox regression model. The numbers of patients at risk after each 12-month interval up to 48 months are indicated below each plot. For patients with Dukes' stage B disease, the survival probabilities at 24 months and 48 months (plus 95% CIs) were 92% (87–97) and 80% (72–87), respectively, for the low suPAR (LO) group and 74% (66–83) and 55% (46–65), respectively, for the high suPAR (HI) group. For patients with Dukes' stage C disease, the survival probabilities at 24 months and 48 months (plus 95% CIs) were 77% (69–85) and 54% (44–64), respectively, for the low suPAR (LO) group and 51% (40–63) and 34% (23–45), respectively, for the high suPAR (HI) group.

and age, as well as CEA dichotomized by its median level (3.8 ng/mL) and suPAR analyzed continuously as the log of suPAR concentration (i.e., ln suPAR). The results are summarized in Table 2. Dukes' stage was statistically significantly associated with survival; patients with Dukes' stage D disease had an HR of 7.5 (95% CI = 5.6–10.2) compared with those with Dukes' stage B disease, and patients with Dukes' stage C disease had an HR of 2.2 (95% CI = 1.6–3.0) compared with those with Dukes' stage B disease. Age scored in years at entry was statistically significantly asso-

ciated with survival (*P* = .02), whereas serum CEA (*P* = .18) and sex (*P* = .19) were not. However, it was also notable that in the multivariate analysis high levels of plasma suPAR were found to be an independent prognostic indication for shorter overall survival, with an HR of 1.9 (95% CI = 1.4–2.5; *P*<.0001) for an increase in ln suPAR of 1.0 or a 2.7-fold increase in suPAR concentration.

DISCUSSION

To our knowledge, this is the first report showing an association between

plasma suPAR levels and survival in patients with colorectal cancer. The results from this retrospective study provide evidence that patients with higher preoperative plasma suPAR levels have a shorter overall survival. Moreover, the data indicate that there is a continuously increasing risk of mortality associated with increasing suPAR level. Multivariate analysis of survival showed that the plasma level of suPAR was a significant prognostic variable independent of Dukes' stage and level of CEA, a molecule previously found to have both a prognostic impact and a value as a marker of early relapse of colorectal cancer *(30–32)*. By use of an arbitrary cut point for testing the relationship to prognosis in each Dukes' stage, the level of suPAR was found to be a statistically significant prognostic variable for patients with Dukes' stage B, C, or D disease. Comparing patients with Dukes' stage B disease with an age- and sex-matched cohort from the general Danish population, we found that patients with Dukes' stage B disease who had low plasma suPAR levels had a survival probability indistinguishable from that of the cohort, whereas patients who had higher plasma suPAR levels had an HR of 2.8. However, we stress that determination of clinically useful cut points, if they exist, clearly requires further study with sufficient validation. Moreover, the patients in this study may not necessarily represent a random sample from the population; therefore, it cannot be concluded that surgically treated patients with Dukes' stage B colorectal cancer who have low suPAR levels have no survival disadvantage.

In the group with Dukes' stage C disease, and even more so in the group with Dukes' stage D disease, almost all patients (and thus all suPAR levels) were found to have a greater risk than their respective matched population cohorts. The difference in this regard between patients with Dukes' stage B disease and patients diagnosed with more advanced disease implies that the preoperative plasma level of this functional marker can be a useful measure of the invasive potential of the tumor (and thus survival) in only early stage disease, since survival at later stages of tumor dissemination is predominantly determined by the difficulty experienced in complete surgical removal of the patient's tumor. The potential value of suPAR as a clinical marker is therefore likely to be greatest in managing the dis-

ease in patients with Dukes' stage B, where the cancer is still potentially curable by surgery but the expected mortality is statistically significant. Plasma suPAR levels could be used in identifying those patients with Dukes' stage B disease who are at high risk and who, therefore, can potentially benefit most from adjuvant treatment. Conversely, plasma suPAR levels could be used to identify patients with Dukes' stage B disease who should not be subjected to adjuvant treatment because they do not have a significantly increased risk from their disease. *Note* that none of the patients in this study received adjuvant therapy.

This study was based on a newly developed suPAR kinetic ELISA method that has a high level of sensitivity and specificity in the measurement of suPAR levels in plasma and serum *(24)*. The results are consistent with those of previous studies performed on extracts of resected tumor tissue from colorectal cancer *(20)*, lung cancer *(19)*, and breast cancer *(21)*; those studies demonstrated that high levels of uPAR in the tumor tissue were related to poor patient survival. Furthermore, we found earlier that, in patients with non-small-cell lung cancer *(23)* and in patients with advanced breast cancer *(24)*, colorectal cancer *(24)*, and ovarian cancer *(25)*, plasma concentrations of suPAR are statistically significantly increased compared with those in healthy individuals. This increase is most likely the result of enzymatic cleavage of uPAR from the surface of tumor cells and/or stromal cells. In colon cancer, uPAR messenger RNA expression and immunoreactivity are enhanced in both tumor cells and tumor-infiltrating macrophages *(33)*.

In conclusion, invasion and metastasis are dependent on proteolytic activity in tumors; accordingly, a large number of studies involving different cancer types, including colorectal cancer, have shown that tumor tissue levels of uPA, uPAR, and type 1 plasminogen activator inhibitor are associated with shorter survival *(19–21)*. Our present findings on plasma suPAR levels in patients with colorectal cancer add new data to these studies and strengthen the view *(25,32,34)* that measurement of functionally relevant blood parameters before resection of a primary tumor may provide valuable prognostic information for patients with cancer. The potential for application of suPAR as a useful clinical marker remains to be de-termined, but this should be sought in the setting of early stage disease.

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