Analysis of Specific Transcriptional Regulators as Early Predictors of Independent Prognostic Relevance in Resected Colorectal Cancer

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Abstract

Purpose: Prognostic studies on transcription factors acting at specific promoter elements have never been done so far. However, in tumors with long necessary follow-up, such as colorectal cancer, early-risk predictors would be needed. The invasion-related gene *u-PAR* is regulated via an activator protein 2 (AP-2)/Sp1 (−152/−135) and an AP-1 binding promoter motif (−190/−171), mediating *u-PAR* induction by K-Ras and Src. The present study was done to give first evidence for early prognostic relevance of transcription factors differentially bound to the *u-PAR* promoter, and their molecular inducers, in colorectal cancer.

Experimental Design: Tumor/normal tissues of 92 prospectively followed (median = 26.3 months) patients were analyzed for Src activity/protein, *K-ras* mutations, and transcription factor binding to both *u-PAR* promoter motifs (*in vivo* gel shift, kinase assay, and PCR).

Results: Kaplan-Meier/Mantel-Cox analysis showed a significant correlation among elevated Sp1/Sp3 binding to region −152/−135 ($P = 0.002$ and $P = 0.006$), the combinations of Sp1/AP-2 and Sp1/AP-1 binding to both motifs ($P = 0.010$ and $P = 0.005$), and Sp1 binding/high Src protein in tumors ($P < 0.001$), with poor survival. Survival decreased with the number of bound transcription factors to both motifs, with binding of three factors defining a high-risk group ($P = 0.021$). In multivariate analysis, elevated Sp1 binding, combinations of Sp1/AP-2 binding and Sp1/AP-1 binding, or Sp1 binding/high Src were independent prognostic variables; u-PAR expression itself being not yet prognostic. A first molecular staging model (CART) was defined, providing novel early high-risk groups (mean survival time as low as for non-curatively resected patients) from these variables.

Conclusions: This study defines transcription factors acting at specific promoter elements of an invasion-related gene, mediating specific signaling, as novel, independent, early predictors of prognosis in colorectal cancer.

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Transcription factors mediate regulatory cascades at specific promoter elements of defined genes. Therefore, although ubiquitously expressed, transcription factors induce differing gene expression and phenotype in different tissue entities. This property could explain why studies have seldom found expression of transcription factors at the protein level to be relevant for clinical prognosis (1–3). Clinical prognostic research on transcriptional regulators bound to specific promoter motifs has not yet been done and has suffered from inadequate cohorts partly due to the challenge of combining molecular methodology with sufficient high-quality resected tissue.

However, because transcription factors are among the earliest regulators of gene expression, one could speculate that, if measured appropriately at specific target genes critical for tumor progression, they might serve as early predictors of prognostic risk. This would be extremely helpful especially for the early prediction of risk groups in tumor types with relatively long median overall survival, such as colorectal cancer. Here, variables predicting prognostic risk even after a short median follow-up would be ideal to contribute to, for example, adjuvant therapy decisions.

We recently showed that the binding of transcription factors to two promoter motifs of the urokinase receptor (*u-PAR*) gene

in colorectal tumor tissue can differ significantly from that in normal tissue from the same patient (4, 5). u-PAR, a M_r 55,000 to 60,000 cell surface receptor, together with its ligand urokinase-type plasminogen activator (u-PA) and a specific inhibitor (PAI-1), comprise the "u-PA system," which has been shown to be essential for tumor invasion and metastasis by inducing plasmin-mediated degradation of extracellular matrix components (6–13). Components of the u-PA system are overexpressed in diverse human tumors, such as breast and gastrointestinal cancers (14–22), and are independently associated with poor prognosis in studies with considerable median follow-up times. High u-PAR expression in cancer is primarily attributable to transcriptional activation of the gene, although mRNA stability or posttranslational modifications represent additional control mechanisms (7, 10, 22–26). Our previous *in vitro* work showed (27, 28) that a motif (−152/−135) of the *u-PAR* promoter, bound with Sp1, Sp3, and an activator protein 2α (AP-2α)–related protein, is an essential mediator of a highly constitutive and phorbol 12-myristate 13-acetate–induced *u-PAR* gene expression. Notably, Sp1 transcription factor bound to this motif was essential for induction of *u-PAR* gene expression and u-PAR–mediated invasion by Src, a cytosolic tyrosine kinase shown to be highly active and prognostic in a preliminary clinical study, in colorectal cancer (28, 29). Our first translational study (4) confirmed these results *in vivo*; binding of transcription factors to this motif was corroborated in resected gastrointestinal carcinoma tissues, and a significant proportion of patients exhibited increased transcription factor binding to this motif in tumors, as opposed to normal tissue. Another *u-PAR* promoter region synergistic with region −152/−135 (27), spanning bp −190/−171 and containing an AP-1 consensus motif, was found to mediate constitutive and phorbol 12-myristate 13-acetate–inducible gene expression (30) and induction of *u-PAR* gene expression via the mitogen-activated protein kinase and c-Jun NH_2-terminal kinase pathway (25, 31). Most importantly, it was required for induction of *u-PAR* gene expression and invasion caused by mutation-activated K-Ras in colon cancer (32). Our recent translational study found that AP-1 family members showed increased binding to this region in resected tumors of a subgroup of colorectal cancer patients compared with corresponding normal mucosa (5), albeit in a smaller number of patients than observed for the Sp1/AP-2 region. Such studies suggest that diverse transcription factors bound to two different *u-PAR* promoter regions, and important molecular pathways mediated by them, such as Src- or K-Ras–induced pathways, are differentially employed in tumor and normal tissues and could be potentially used as tumor-selective targets for countering invasion and metastasis in certain cancer patient subgroups. However, up to now, no prognostic study has been done for these *u-PAR* promoter–related pathways to support such a clinical notion, nor has any prognostic study been done for transcription factor binding to specific promoter sites in general.

Therefore, we did this comprehensive prognostic study on transcription factors specifically bound to *u-PAR* promoter regions −152/−135 and −190/−171 as essential mediators of diverse pathways of u-PAR control on a series of 92 prospectively followed colorectal cancer patients. Specifically, the present study should explore whether transcription factors bound to the *u-PAR* promoter can serve as early predictors of high prognostic risk. Because their influence on *u-PAR* gene expression is mediated by these specific transcriptional regulators, Src-activity and presence of *K-ras* exon 1 mutations were also analyzed in this cohort to identify patient subgroups with clinically relevant pathway differences inducing u-PAR–mediated invasion. This is the first clinical study suggesting Sp1 in particular (but also transcription factor combinations involving the AP-2–like protein and AP-1 and Sp1 binding in combination with high Src) as a new and independent early risk factor for survival in resected colorectal cancer, thus defining new prognostic high-risk groups in an extended molecular staging model, at an early time of follow-up observation.

Materials and Methods

Patients and tumors. Ninety-two prospectively followed patients underwent surgery for primary colorectal cancer between March 1999 and October 2003. Twenty were included in the series of Schewe et al. (5). Patient and tumor characteristics are summarized in Table 1. Follow-up (physical exam, ultrasound, chest X-ray, and tumor markers CEA and Ca 19-9) was carried out 6, 12, 18, and 24 months after surgery and at 1-year intervals thereafter. Adjuvant therapy was administered to 17 patients. Tumor recurrence was diagnosed by biopsy or explorative surgery. Causes of death were evaluated clinically. The study was approved by the institutional ethics committee and done with patients' informed consent. Tissue specimens (tumor and normal mucosa) were collected after verification by a pathologist and immediately frozen in liquid nitrogen. Analyses were done without knowledge of the patients' identity, tumor stage, etc.

Preparation of nuclear extracts. Preparation of nuclear extracts was done essentially as described in our previous publications (4, 5).

Electrophoretic mobility shift assay and supershift analysis. Our previously established *in vivo* gel-shift and supershift method (4, 5) was used; it yielded clear, reproducible transcription factor binding in resected tissue. The identity of bound transcription factors was verified by supershifting, or competition with a consensus oligonucleotide, as published previously (4, 5).

ELISA. To determine u-PAR protein amounts. u-PAR protein in tissue cytosols was assayed using IMUBIND total u-PAR ELISA kit (American Diagnostica, Greenwich, CT).

DNA isolation and PCR. For the isolation of genomic DNA, the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) was used. A 251-bp DNA fragment, including *K-ras* exon 1, was amplified using 1 μg of genomic DNA, Pwo polymerase, and the primers forward, 5′-GGTAC-TGGTGGAGTATTTGATAGTGTATTAACC-3′ and reverse, 5′-GAATGG-TCCTGCACCAGTAATATGC-3′ (initial denaturation, 94°C for 3 min; 40 cycles: denaturation, 94°C for 30 s; annealing, 62°C for 30 s; elongation, 72°C for 45 s; prolonged elongation, 72°C for 5 min). PCR products were purified (QIAquick PCR purification kit, Qiagen) and sequenced (ABI 3700 Capillary Sequencer, Applied Biosystems, Foster City, CA). A water sample and HCT116 (cell line with a heterocygote mutation in *K-ras* codon 13) served as controls.

Src kinase assay. Thirty microliters of protein G-Sepharose (Amersham, Piscataway, NJ) and 1 μg anti-Src (Oncogene, Cambridge, MA) were added to 500 μg of cytosolic protein for immunoprecipitation and incubated at 4°C for 4 h. After centrifugation, 20 μL buffer [0.5 μg/μL myelin basic protein, 6 μCi γ-[^{33}P]ATP, 20 mmol/L HEPES (pH 7.2), 1 mmol/L $MnCl_2$, 1 mmol/L DTT, 0.1 mmol/L sodium orthovanadate] were given to the pellet and shaken for 10 min at 37°C. The reaction was terminated by 20 μL Laemmli buffer(2×); samples were boiled for 4.5 min at 95°C and subjected to Tris-glycine SDS-PAGE (10%; running buffer: 25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS). After semi-dry blotting (0.5 mA/cm^2, 60 min) and staining with Ponceau S, gels were exposed to Kodak-BioMax-MR films and quantified using phosphoimaging. As controls, cell lines RKO (intermediate Src activity) and 2C8

Table 1. Patient and tumor characteristics of the colorectal patient series (N = 92)

	Absolute	Relative (%)
Sex		
Male	63	68.5
Female	29	31.5
Localization		
Caecum/colon ascendens	21	22.8
Colon transversum	18	19.6
Colon descendens/sigmoideum	25	27.2
Rectum	28	30.4
Intention of treatment		
Curative	68	73.9
Palliative	24	26.1
Resection		
R0	68	73.9
R1	2	2.2
R2	22	23.9
UICC classification		
Ia	2	2.2
Ib	10	10.9
IIa	33	35.9
IIb	2	2.2
IIIb	13	14.1
IIIc	5	5.4
IV	27	29.3
Dukes classification		
A	12	13.0
B	35	38.0
C	18	19.6
D	27	29.3
Grading		
G1	4	4.3
G1-G2	1	1.1
G2	50	54.3
G2-G3	10	10.9
G3	27	29.3
Staging, tumor size (pT)		
pT_1	2	2.2
pT_2	11	12.0
pT_3	62	67.4
pT_4	17	18.5
Staging, affection of lymph nodes (pN)		
pN_0	52	56.5
pN_1	21	22.8
pN_2	19	20.7
Staging, metastases (M)		
M_0	65	70.7
M_1	27	29.3
Lymphangiosis carcinomatosa		
No	47	51.1
Yes	45	48.9

(high endogeneous Src activity; ref. 28) were used in every experiment. Western blotting for β-actin as control and Src protein was done as described in ref. (28).

Quantification of results. Binding activities of AP-1, Sp1, Sp3, and the AP-2α–related factor in tumor as well as normal tissues and in a standardized control (RKO) were quantified as previously described (4, 5) with the modification of using phosphoimaging (Fuji BAS-1800 II) and AIDA software, version 3.44. In Src kinase assays, autophosphorylation and phosphorylation of the substrate myelin basic protein were quantified using phosphoimaging/AIDA, using 2C8 lysates as a standardized control. Specific Src activity was calculated in reference to Src protein as measured in parallel (Western; ref. 28). Signal intensities minus background were calculated and expressed relative to the 2C8 value (kinase assays) or the RKO band intensities (electrophoretic mobility shift assays). Elevated transcription factor binding or elevated Src kinase activity was defined as at least 1.5-fold in tumor tissue compared with normal mucosa because this cutoff discriminated the two groups best in CART analysis.

Statistical analysis. Analysis was done using SPSS version 13.0 (SPSS Inc., Chicago, IL). The two-sided significance level was $\alpha = 0.05$; a statistical trend was defined as $\alpha \leq 0.1$. Differences of quantitative variables between tumor and normal tissues were tested using the two-sample paired Wilcoxon test. The Mann-Whitney test was used to compare patient subgroups for quantitative variables. Spearman correlations among continuous variables were computed. χ^2 tests (Bonferroni corrected) were applied for grouped/dichotomized variables; survival was estimated by Kaplan-Meier analysis, and differences were tested by Mantel-Cox log-rank statistics; the primary end point was tumor-related death (disease-specific survival). Multivariate analysis, including established clinical risk factors in colorectal cancer, was done according to the Cox proportional hazard model. The following variables were dichotomized: pT stage as pT_1/pT_2 versus pT_3/pT_4, involved lymph nodes as pN_0 versus pN_1-pN_3, distant metastasis as M_0 versus M_1, histologic grading as G_1/G_1-G_2/G_2 versus G_2-G_3/G_3, lymphangiosis carcinomatosa as present versus absent. As done previously by our group (33), stepwise CART regression tree analysis yielded a first molecularly extended staging model, allowing stepwise prognostic subgroup identification.

Results

Patient results. Ninety-two patients (68 R0 resected; Table 1) with primary colorectal cancer were analyzed for transcription factors bound to regions −152/−135 and −190/−171 of the *u-PAR* promoter, endogenous Src activity and Src protein levels (Fig. 1), and *K-ras* mutations (codon 12/13; Table 2), in primary tumors and corresponding normal mucosae, as main regulators of the *u-PAR* as defined previously (27, 28). Median follow-up was 26.3 months (range, 0.1-71.6). Local tumor recurrence/metastasis was observed in 10 R0-resected patients; a total of 17 patients died due to the tumor.

Descriptive analysis for Src activity/protein, K-ras mutations, and transcription factor binding to u-PAR promoter motifs. Median u-PAR protein level in 92 patients was 2.94 ng/mg protein (range, 0.14-16.03) in tumors versus 0.95 ng/mg protein (range, 0.00-3.89) in normal tissue. Differences were significant ($P < 0.001$). Sixty-nine patients from which sufficient tissue was available were investigated for Src activity/protein. Src activity ($P = 0.013$) and Src protein levels were significantly higher in tumors than in normal tissue ($P < 0.001$, Fig. 1C). No correlation between Src activity and Src protein was observed. Elevated Src activity (≥1.5-fold activity in tumors) was found in 43% of patients. Twenty-six *K-ras* exon 1 mutations (29%) were found in 91 pairs of tumor tissue/normal mucosa (Table 2; Supplementary Fig. S1). Modified bases were detected exclusively in tumor samples. All point mutations were localized in codon 12 (65%) and 13 (35%). G/A transitions (54%) outweighed G/T and G/C transversions (39% and 8%, respectively). Tissue was available for application of our previously established *in vivo* gel-shift and supershift method (4, 5) in 90 cases regarding the AP-1 motif and 86 cases regarding AP-2/Sp1 motif. Examples for binding of Sp1, Sp3, and the AP-2–related factor to region −152/−135 and of AP-1 complexes to region −190/−171 of the *u-PAR* promoter are given in Fig. 1A and B and were comparable with our previous studies (4, 5). Elevated binding (>1.5-fold) of AP-1 to region −190/−171 was observed in 38%, of the AP-2–like protein to region −152/−135 in 43%, of Sp1 in 31%, and of Sp3 in 30%

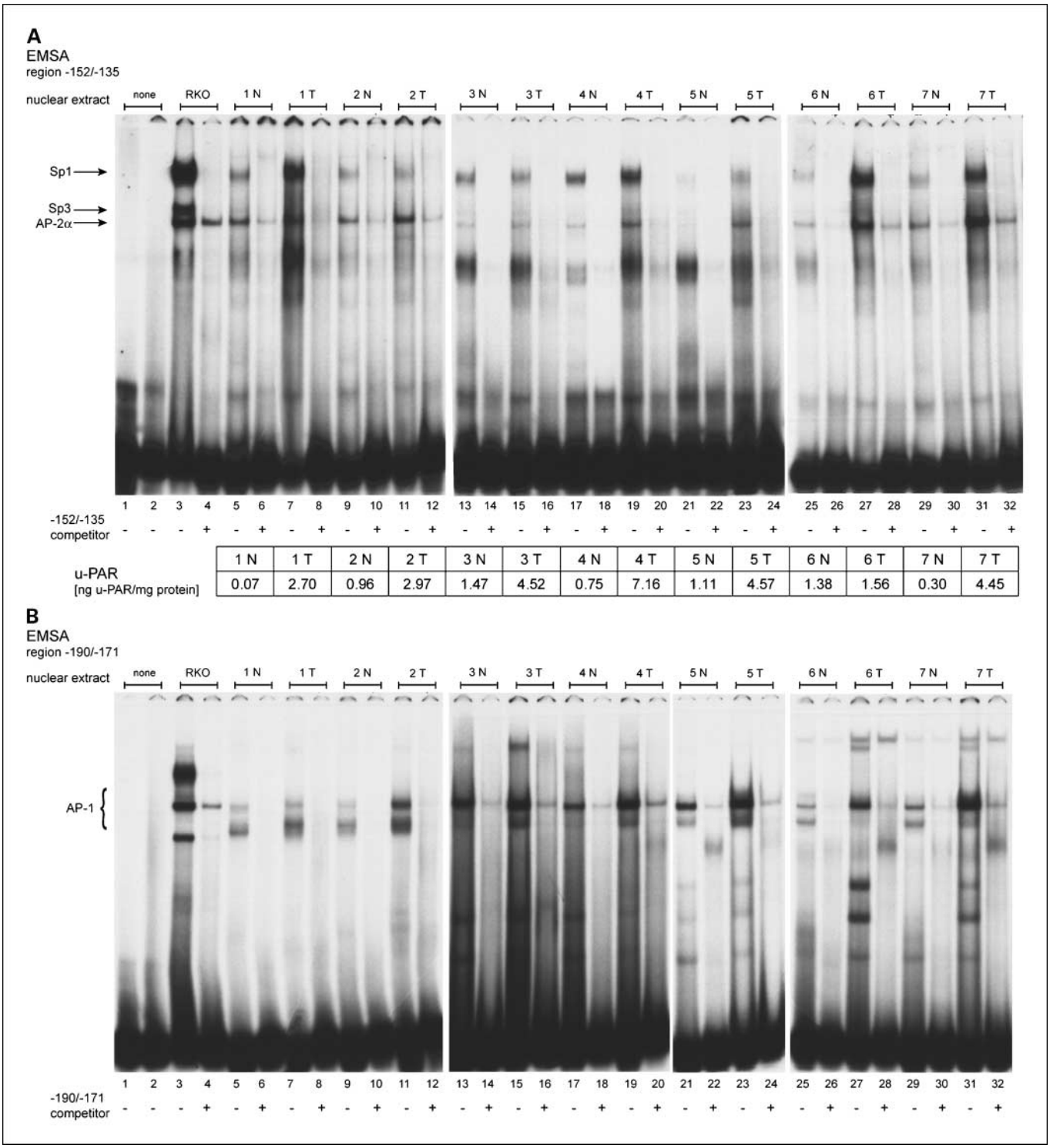

Fig. 1. Examples of electrophoretic mobility shift assays using oligonucleotides for region −152/−135 (*A*) or region −190/−171 (*B*) of the *u-PAR* promoter and of Src kinase assays and Src protein measurements (*C*; *top,* Src kinase assay; *bottom,* Src Western blotting). N, normal mucosa; T, tumor tissue. Corresponding u-PAR protein amounts as measured by ELISA are given below the gel-shift examples of (*A*).

of cases. Binding of AP-1 and of the AP-2–related factor was significantly elevated in tumor tissues compared with normal mucosae ($P < 0.001$ and $P = 0.021$).

Correlation of Src activity, K-ras mutations, and transcription factor binding to region −190/−171 and −152/−135 with established tumor characteristics. To assess the association of all measured molecular regulators with established clinical tumor variables, χ^2 analysis (Bonferroni corrected) was done with variables classified as described in Materials and Methods. Src activity showed a significant correlation with Dukes and

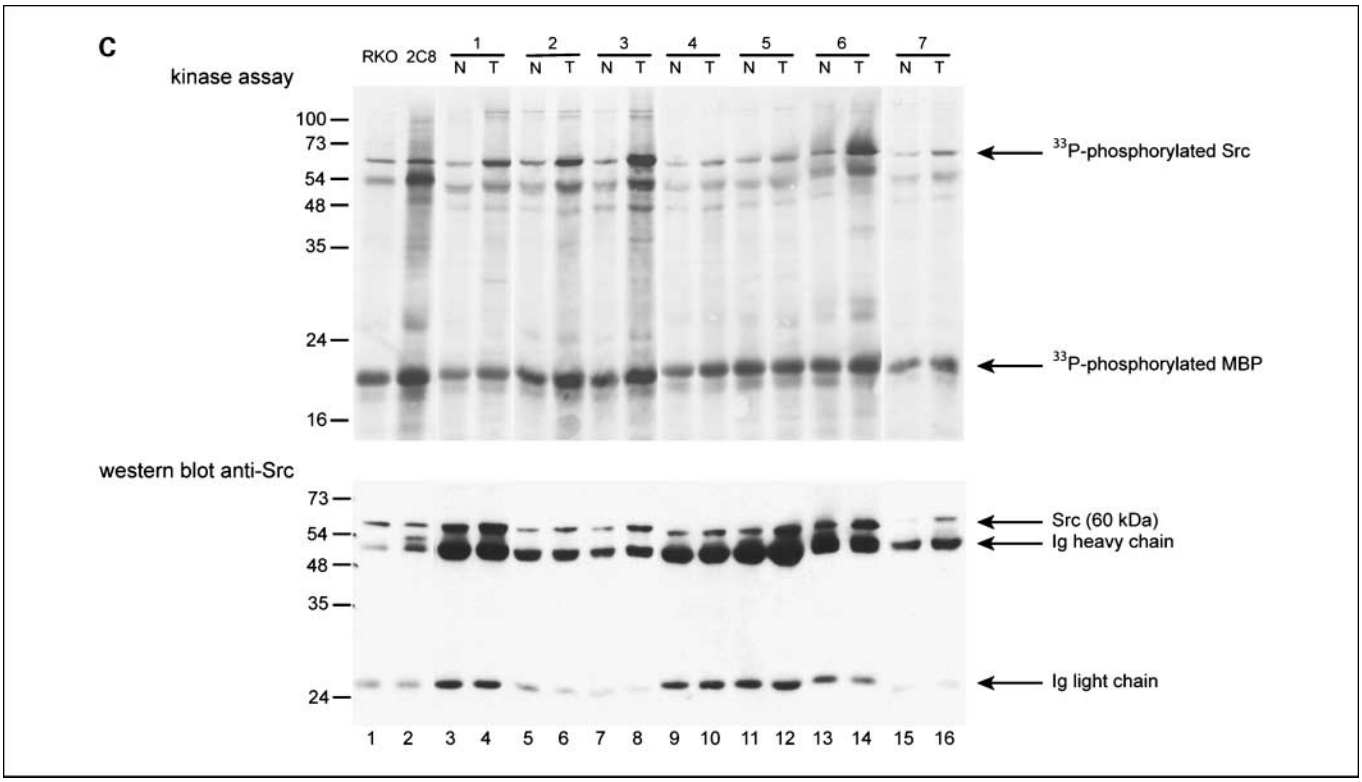

Fig. 1 *Continued.*

Unio Internationalis Contra Cancrum (UICC) stages and with pN ($P = 0.045$, $P = 0.045$, and $P = 0.049$). Elevated Src protein (tumor/normal tissue > 1.5) was observed in poorly differentiated tumors and in tissues with lymphangiosis carcinomatosa as a statistical trend. High u-PAR concentrations were significantly associated with poorly differentiated tumors (G_2-G_3 and G_3, $P = 0.004$). Sp3 bound to the −152/−135 region correlated significantly with pN stage ($P = 0.030$). There was a trend for Sp3 to correlate with M and Dukes stage and for Sp-1 bound to this region to correlate with M ($P \leq 0.1$).

Correlations among Src activity, Src expression, K-ras mutations, u-PAR, and transcription factor binding to u-PAR promoter motifs in resected tissues. Next, we considered correlations between transcription factors and further molecular regulators of the u-PAR in resected tissues of the present patient series, to support pathways previously shown in cell lines. Binding of Sp1 and Sp3 to *u-PAR* promoter region −152/−135 correlated with u-PAR protein ($P = 0.005$ and $P = 0.002$); AP-2 binding to the same region correlated as a trend. AP-1 binding to *u-PAR* promoter region −190/−171, Src activity and Src protein correlated significantly with high u-PAR ($P = 0.001$, $P = 0.034$, and $P = 0.028$, respectively). Exclusively in tumor samples, Src protein levels correlated positively with binding of Sp1, Sp3, and the AP-2–related protein to *u-PAR* region −152/−135 ($P = 0.004$, $P = 0.010$, and $P = 0.031$). High Src activity was positively associated with transcription factor binding to the AP-1 motif, in tumor and normal tissue ($P < 0.001$). These correlations give *in vivo* support particularly for the pathways shown for Src and Sp1/AP-1 transcription factors in u-PAR regulation (28, 34). No significant association was observed between *K-ras* mutations and Src protein levels. Src activity was significantly higher in tumors without mutated *K-ras* compared with tumors presenting a *K-ras* mutation ($P = 0.019$). These results suggest that K-Ras– and Src-induced mechanisms regulating u-PAR might be independent in individual resected colorectal cancers.

Table 2. Number, type, and frequency of the different mutations detected in *K-ras* exon 1 and corresponding amino acids

Codon (wild type, amino acid)	Type of mutation	Frequency absolute (relative, percentage of all mutations detected)	Mutated codon (amino acid)
12 (GGT, glycine)	G→A	5 (19.2%)	GAT (aspartic acid)
		1 (3.8%)	AGT (serine)
	G→T	10 (38.5%)	GTT (valine)
	G→C	1 (3.8%)	GCT (alanine)
13 (GGC, glycine)	G→A	8 (30.8%)	GAC (aspartic acid)
	G→C	1 (3.8%)	CGC (cysteine)

Univariate prognostic effect of molecular u-PAR regulators and their combinations. In univariate analysis for all measured molecular u-PAR regulators, the presence of *K-ras* exon 1 mutations was not significantly associated with disease-specific survival. u-PAR protein expression itself was associated with disease-specific survival in trend only ($P < 0.1$). Nevertheless, in Kaplan-Meier analysis (Mantel-Cox log-rank, median follow-up of 26.3 months), elevated Sp1 binding in tumor tissue to *u-PAR* promoter region −152/−135 was significantly associated with a shorter disease-specific survival (end point tumor-related death, $P = 0.002$; Fig. 2A). Sp3 bound to the same region correlated significantly with a shorter disease-specific survival ($P = 0.006$; Supplementary Fig. S2A) and with shorter recurrence-free survival ($P = 0.042$). The AP-2–related factor was associated with a poor survival in trend ($P = 0.063$). Neither elevated Src activity nor Src protein reached significance taken alone. However, the combination of high Src protein level and elevated Sp1 binding to *u-PAR* promoter motif −152/−135 exhibited the strongest univariate association with poor recurrence-free ($P = 0.016$) and disease-specific ($P < 0.001$) survival (Fig. 2B).

In addition, the combinations of elevated Sp1 binding and the AP-2–like transcription factor to region −152/−135, Sp1 binding at −152/−135 and AP-1 binding to region −190/−171 (Supplementary Fig. S2B and C) and Sp-1/Sp3 binding to region −152/−135 were significantly associated with disease-specific survival ($P = 0.010$, $P = 0.005$, and $P = 0.006$). Interestingly, in Kaplan-Meier analysis, we were able to define an increasing clinical risk depending on the number of transcription factors bound. As can be seen from Fig. 2D, a gradually poorer disease-specific survival with increasing numbers of bound transcription factors to these *u-PAR* promoter motifs can be found, with binding of three transcription factors (AP-1, Sp1, and the AP-2–like protein) significantly defining a high risk group for overall survival ($P = 0.021$).

Multivariate analysis of molecular u-PAR regulators and definition of a first molecular staging model. Finally, we did

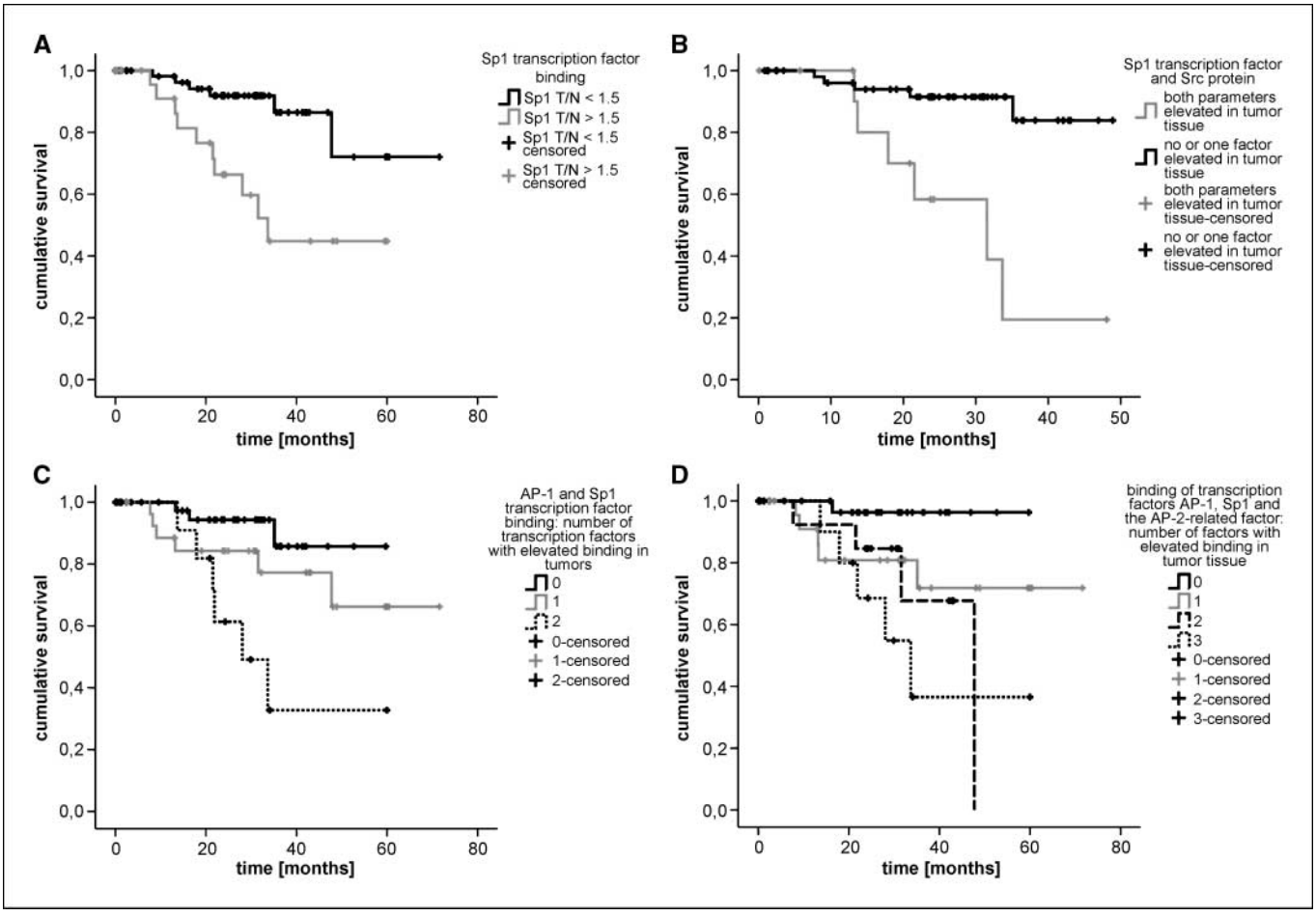

Fig. 2. *A,* disease-specific survival (86 patients) depending on Sp1 binding to the −152/−135 *u-PAR* promoter region: ratio of tumor to normal tissue (*T/N*). $P = 0.002$ (Mantel-Cox). MST, mean survival time. Sp1 *T/N* ‹ 1.5: 59 cases, 6 events, MST of 61.6 mo, SE 4.1; Sp1 *T/N* › 1.5: 27 cases, 10 events, MST of 38.7 mo, SE 4.8. *B,* disease-specific survival (69 patients) for patients showing both elevated binding of Sp1 and elevated Src protein concentration (group 2) and for patients showing no elevated binding and/or protein amount (group 1). $P < 0.001$ (Mantel-Cox). Group 1: 56 cases, 5 events, MST of 44.8 mo, SE 1.7; group 2: 13 cases, 6 events, MST of 29.0 mo, SE 4.2. *C,* disease-specific survival (85 patients) according to the presence or absence of elevated binding of the transcription factors AP-1 and Sp1. $P = 0.005$. AP-1 and Sp1 *T/N* ‹ 1.5: 41 cases, 3 events, MST of 55.1 mo, SE 2.6; AP-1 or Sp1 *T/N* › 1.5: 29 cases, 6 events, MST of 56.4 mo, SE 5.3; AP-1 and Sp1 *T/N* › 1.5: 15 cases, 6 events, MST 35.9 mo, SE 6.1. *D,* disease-specific survival (85 patients) according to the number of transcription factors showing elevated binding to *u-PAR* promoter elements −190/−171 and −152/−135. $P = 0.021$. None: 30 cases, 1 event, MST of 58.1 mo, SE 1.6; one: 27 cases, 5 events, MST of 56.7 mo, SE 5.8; two: 15 cases, 4 events, MST of 39.9 mo, SE 4.6; three: 13 cases, 5 events, MST of 37.6 mo, SE 6.5.

Table 3. Multivariate analysis of molecular u-PAR regulators

Variable	*P*	HR (95% CI)
Elevated Sp1 binding	0.005	4.98 (1.61-15.39)
Resection	0.031	4.70 (1.15-19.19)
UICC stage	0.005	14.45 (2.21-94.60)
Elevated binding of Sp1 and the AP-2 – related factor	0.021	2.15 (1.12-4.12)
Resection	0.018	5.58 (1.34-23.18)
UICC stage	0.013	8.91 (1.59-50.06)
Elevated binding of AP-1 and Sp1	0.007	2.74 (1.31-5.71)
Resection	0.019	5.62 (1.34-23.64)
UICC stage	0.010	9.81 (1.72-55.89)
Elevated Src-protein and elevated Sp1-binding	0.025	4.23 (1.20-14.90)
Resection	0.001	31.44 (3.78-261.31)
Elevated binding of AP-1, Sp1, and the AP-2 – related factor	0.018	1.83 (1.11-3.02)
Resection	0.018	5.73 (1.35-24.29)
UICC stage	0.018	7.85 (1.42-43.36)
Elevated binding of AP-1, Sp1, Sp3, and the AP-2 – related factor	0.021	1.57 (1.07-2.30)
Resection	0.031	4.94 (1.16-21.08)
UICC stage	0.014	8.78 (1.56-49.37)

Note: u-PAR regulators include disease-specific survival, resection: curative versus non-curative resection, elevated binding: binding at least 1.5 times higher in tumor tissue than in corresponding normal mucosa, UICC stage: UICC stage dichotomized, ≤IIb versus ≥IIIa

multivariate analysis considering established clinical risk factors. Variables were included in the Cox proportional hazard model if they had shown univariate significance. Table 3 summarizes the significant factors in the multivariate models. As key results, elevated Sp1 binding to *u-PAR* promoter region −152/−135 in resected colorectal tumor tissue was a new, independent prognostic factor for disease-specific survival [P = 0.005; hazard ratio (HR), 4.98; 95% confidence interval (95% CI), 1.61-15.39]. Surgical curability (curative versus non-curative resection) and UICC stage also entered the model. Elevated binding of the combination of Sp-1 and the AP-2 – like protein to *u-PAR* promoter region −152/−135 was also a new and independent risk factor (disease-specific survival: P = 0.021; HR, 2.15; 95% CI, 1.12-4.12), again in addition to surgical curability and UICC. Moreover, the combination of AP-1 binding to region −190/−171 and Sp1 binding to region −152/−135 was independently associated with poor disease-specific survival (P = 0.007; HR, 2.74; 95% CI, 1.31-5.71), besides UICC and surgical curability, and with poor recurrence-free survival, besides UICC (P = 0.037; HR, 2.61; 95% CI, 1.06-6.41). Furthermore, the combination of elevated Src protein in tumor tissue and Sp1 binding to *u-PAR* promoter motif −152/−135 was an independent predictor of disease-specific survival (P = 0.025; HR, 4.23; 95% CI, 1.20-14.90) in addition to surgical curability and of recurrence-free survival (P = 0.021; HR, 6.51; 95% CI, 1.33-31.92) in addition to UICC. Finally, the binding of three transcription factors (AP-1, Sp1, AP-2α: P = 0.018; HR, 1.83; 95% CI, 1.11-3.02) and the binding of all four transcription factors (AP-1, Sp1, Sp3, and AP-2α) were independent predictors of disease-specific survival, besides surgical curability and UICC (P = 0.021; HR, 1.57; 95% CI, 1.07-2.30).

Finally, we sought to define a first molecularly broadened clinical staging model in our colorectal cancer patient series according to our previous strategy (33). Figure 3 shows a stepwise regression tree, starting with the most significant clinical risk factor surgical curability (R). Within curatively (R0) resected patients, the UICC stage can separate additional significant prognostic subgroups as the second most powerful variable (Fig. 3). However, in all patients, additional prognostic high-risk groups can be defined resulting from elevated binding of transcription factors to specific *u-PAR* promoter motifs in resected tumors. For example, elevated Sp1 binding within the subgroup of advanced UICC stages in R0-resected patients predicts a prognosis almost as poor as the prognosis of non-curatively resected patients, as judged from median disease-specific survival time (25.9 versus 21.4 months; Fig. 3), with prognosis of this group being significantly different from predictions by UICC alone (Fig. 3). An analogous high-risk group could be defined by high AP-1 binding within R0-resected patients with advanced UICC stages ($P_{\text{AP-1 high/low}}$ = 0.010). This analysis shows that it is possible to select new high-risk groups for disease-specific survival in curatively resected patients, from specific transcriptional regulators at the promoter of a gene supporting metastatic progression and this already at a rather early time point of median follow-up observation.

Discussion

This study implicates in particular Sp1 bound to *u-PAR* promoter region −152/−135 but also transcription factor combinations involving the AP-2 – like protein bound to the same promoter motif, or AP-1 bound to *u-PAR* promoter motif −190/−171, as new and independent early risk factors for disease-specific survival in resected colorectal cancer. In addition, elevated Sp1 binding in combination with high Src protein in tumor tissue defines a prognostic high-risk group. Most notably, we found that the clinical prognostic risk increases progressively with the number of transcription factors bound in tumor tissues compared with normal tissues. This supports the hypothesis that clinical risk increases with increasing numbers of activated molecular/transcriptional pathways towards a gene critical for tumor progression. The fact that transcriptional regulators contribute independent prognostic information and allow the definition of a molecularly

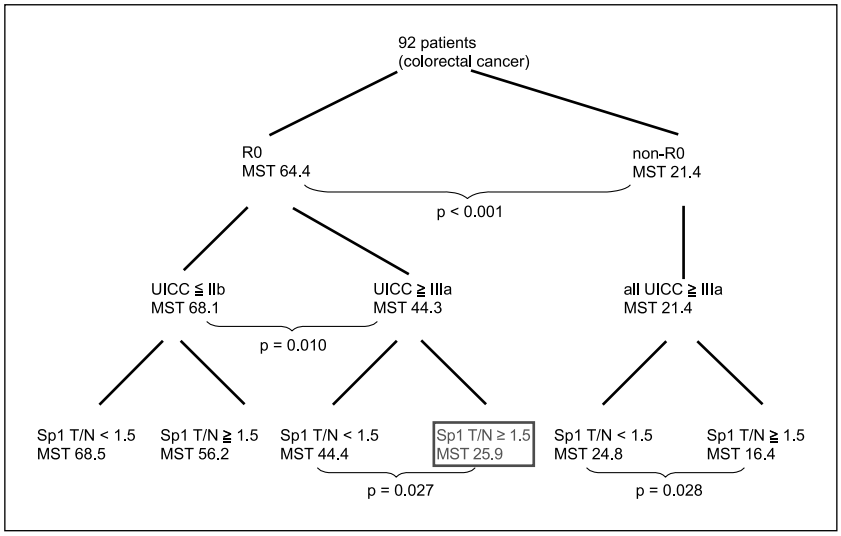

Fig. 3. Example of regression tree analysis to define a first molecular staging model, including transcription factor binding to the *u-PAR* promoter. Sp1 in this example significantly defines a new high-risk group within R0-resected patients with advanced UICC stages; mean survival time in this group was almost as poor as in R1/2-resected patients.

broadened staging model at a rather early time of median follow-up (26.3 months) suggests that measurements of critical transcriptional regulators of progression-related genes, such as *u-PAR*, might provide earlier prognostic information than gene expression measurements per se.

Correlations and prognostic models presented here corroborate previous *in vitro* findings on pathways regulating *u-PAR* gene expression and invasion, by new *in vivo* data from resected tissues. Sp1 bound to promoter region −152/−135 had been shown to mediate a specific induction of *u-PAR* gene expression and invasion via Src in cultured colon cancer (28), this pathway now being corroborated by a significant correlation of Sp1 binding and Src and by the fact that the combination of both variables confers a poor prognosis to colorectal cancer patients. The AP-2–like protein bound to the same promoter motif had been shown to mediate a high constitutive and phorbol 12-myristate 13-acetate–inducible *u-PAR* gene expression, with the present study identifying a prognostic high-risk group for transcription factor combinations involving the AP-2–like protein. Other *in vitro* studies showed that other invasion-related genes, such as *MMP-2*, *MMP-9*, *cathepsin D*, *cathepsin B*, and *MT1-MMP*, are regulated by AP-2–like transcription factors or/and Sp1 (in part also Sp3); this is in part via combined AP-2/Sp1/(Sp3) promoter motifs (31, 35), confirming an important role of these two transcription factors and their interaction in tumor invasion and progression. In addition, the AP-1 family of transcription factors is known to promote proliferation, tumor cell progression, invasion, and metastasis, besides *u-PAR* regulating other metastasis-related genes such as *PAI-1*, *u-PA*, *MMP-3*, *MMP-9*, and *VEGF* (36–38). In particular, the AP-1 motif −190/−171 of the *u-PAR* promoter had been shown to be a mediator of many general signal transduction pathways leading to constitutive *u-PAR* gene expression, including the Ras/mitogen-activated protein kinase and the c-Jun NH_2-terminal kinase pathway (25, 30). Our yet unpublished data also suggest that this AP-1 motif, besides Sp1 bound to motif −152/−135, is a second mediator of Src-induced *u-PAR* gene expression, an observation that is now confirmed *in vivo* by a significant correlation of a high Src-activity with elevated AP-1 binding to *u-PAR* promoter region −190/−171 in resected tumors. Thus, it is not surprising that combinations of Sp-1/an AP-2–like protein, Sp-1/AP-1 bound to the promoter of the *u-PAR* gene, or a combination of Sp1 binding and high Src protein concentration are independently associated with poor clinical prognosis.

Remarkably, our present study showed that, in resected tumors, Src activity was significantly higher in patients without detectable *K-ras* exon 1 mutations in tumor tissue than in patients with *K-ras* mutations. This observation might indicate that mutation-activated K-Ras– and Src-induced signaling is employed independently to induce gene expression in resected colorectal cancers. In the present patient series, K-Ras–induced pathways towards u-PAR might not be as prominent, or prognostically relevant, as Src-induced pathways or other cascades mediated by the transcription factors and promoter motifs analyzed here. For a specific Src activity in tumor tissues presented here, however, a significant correlation was found with pN stage (lymph node involvement), UICC stage, and Dukes stage, suggesting that Src activation clinically might support colorectal cancer progression, especially via lymphatic spread. The strong prognostic association of the combination of high Sp1/high Src particularly supports the notion that Src-induced *u-PAR* gene induction, specifically mediated via Sp1 at this promoter element, is a critical pathway for clinical tumor progression. The additional finding that u-PAR protein levels were significantly higher in low-differentiated colorectal tumors (histologic grade G_2-G_3/G_3) corroborates previous findings of our group (39) and supports the notion that pathways leading to *u-PAR* gene induction might promote cancer dedifferentiation.

There have been previous studies investigating the expression of transcription factors in resected tumor tissues. A recent study of Heimberger et al. (40) suggested that loss of AP-2α expression correlates with the grade of malignant gliomas, though not exhibiting a strong effect on survival. Friedrichs et al. (41) investigated different AP-2 isoforms in breast cancer and revealed different expression patterns in malignant and normal tissue, which, however, were not associated with prognosis. Two studies showed that nuclear localization of AP-2 transcription factors correlated with an unfavorable clinical outcome in prostate and ovarian cancers, with variable effect of this variable in multivariate analysis (1, 2). The study

of Wang et al. (3), who investigated Sp1 expression immunohistochemically in gastric cancers, was one of the few studies thus far to show a significant and independent association with survival. Regarding AP-1, Assimakopoulou and Varakis (42) showed a correlation of c-Jun and c-Fos expression with WHO grade in 80 astrocytomas, suggesting that malignant progression of this entity might be partly due to an increased presence of AP-1. Similarly, Papachristou et al. (43) detected high protein levels of AP-1 in osteosarcoma tissues, with c-Fos and c-Jun expression increasing with tumor grade. In another study (44), AP-1 protein levels increased with malignant progression to laryngeal cancer. However, all of these studies were done exclusively by analyzing transcription factor protein levels by immunohistochemistry/Western. However, it is generally accepted (45) that the biological relevance of a transcription factor is more closely related to its activity and binding to a specific target site than to just its protein expression level. Thus, for example, Sp1 binding to *u-PAR* promoter region −152/−135 in resected tissues was not associated with comparable protein amounts as measured by Western blotting (4). It is well known that phosphorylation, or other posttranslational modifications, are essential for binding and transactivating activities of transcription factors, as shown, for example, for Sp1 or AP-1 (46–49). Thus, studies done just at the protein level are likely to miss functional transcription factor activities in the tissues investigated; this could explain contradictory results thus far on the prognostic effect of their expression.

The present study defines novel, high-risk groups of colorectal cancer patients based on specific molecular and especially transcriptional regulators of the invasion-related gene *u-PAR*, showing independent prognostic relevance of such pathways at quite early time points of clinical follow-up. The study furthermore suggests that specific transcription factors bound to both *u-PAR* promoter elements, and upstream inducers mediated by these transcription factors (such as K-Ras and Src), are differentially activated in tumor and normal tissues of patients, allowing conclusions as to which u-PAR regulatory pathways may be preferred in the individual patient. This strategy in the future may allow the selection of individualized specific molecular targeting approaches (e.g., Src inhibition mediated by region −152/−135) or even transcriptional targeting approaches (50) for individualized inhibition of tumor progression induced by these pathways. From our example showing that molecular/transcriptional pathways can be used to define early molecularly broadened clinical staging models and novel high-risk groups, we hypothesize that further translational approaches will enhance an increasing trend towards individualized diagnosis and targeting, based on the status of specific activities of molecular regulators, in the field of cancer therapy.

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