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Evaluation of *Plakophilin-3* mRNA as a biomarker for detection of circulating tumor cells in gastrointestinal cancer patients

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Abstract

Background: This study aims to assess *Plakophilin-3* (*PKP3*) as a surrogate biomarker of circulating tumor cells in patients with gastrointestinal cancer.

Methods: The primary aim is to estimate the diagnostic accuracy of *PKP3* real-time reverse transcriptase-PCR in blood. Receiver operating characteristic curves were constructed. Correlations between the blood *PKP3* levels and the clinicopathologic features of the study subjects were analyzed. Logistic regression was used to predict outcomes based on *PKP3*.

Results: Sixty-four patients with gastrointestinal cancer and 23 controls were included. The mean relative *PKP3* mRNA expression was 48.45 in cancer patients and 2.8 in controls ($P < 0.0001$). Comparing the *PKP3* levels in patients and controls, the area under the curve was 0.852 (95% confidence interval, 0.76-0.94; $P < 0.0001$) in receiver operating characteristic analysis. A higher blood level of *PKP3* mRNA was associated with a more advanced stage ($P = 0.025$), pT_{3-4} tumors ($P = 0.028$), metastasis ($P = 0.021$), and residual (R2) disease ($P = 0.037$). Higher *PKP3* mRNA was associated with the risk of cancer progression and death (odds ratio, 3.875; 95% confidence interval, 1.781-8.430; $P = 0.001$).

Conclusions: Increased *PKP3* mRNA was detected in the blood of gastrointestinal cancer patients. Significant correlations were found with advanced stage, pT_{3-4}, metastatic disease, and the residual disease status. *PKP3* mRNA in blood was associated with the risk of cancer progression and death.

Impact: PKP3 mRNA can be used as a marker of subclinical disease in gastrointestinal cancer and thus holds potential clinical relevance as a predictor for disease outcome. Cancer Epidemiol Biomarkers Prev; 19(6); 1432–40.

Introduction

Cancers of the gastrointestinal tract are among the most frequent causes of cancer death worldwide (1). The stage at diagnosis and the available options for curative surgery remain the most important prognostic factors. However, distant and locoregional relapses frequently occur despite resection and adjuvant therapy. Circulating tumor cells (CTC) and occult metastasis (i.e., micrometastasis) are considered early events in the multistep process of metastasis (2). Detection of tumor cells in the blood or minimal deposits in distant organs such as the bone marrow and in peritoneal cavity could be important to identify patients at a high risk of disease progression. Sensitive methods for CTC detection could be used as surrogate markers for adjuvant treatment in gastrointestinal cancer patients. Furthermore, characterization of the molecular signature of CTC may help in the selection of new therapeutic targets and in the determination of which patients are suitable for these treatments (3, 4).

Immunologic and molecular approaches are the major methods used to detect isolated tumor cells. PCR amplification of tissue or tumor-specific mRNA is the most powerful tool for the detection of circulating or occult metastatic cells (5). *Cytokeratins 19* and *20*, *CEA*, *guanylyl cyclase C*, *hTERT*, *Ep-CAM*, and *EGFR* are among the mRNA markers used in different reverse transcriptase-PCR (RT-PCR) assays in gastrointestinal cancer patients (6-8). However, genetic heterogeneity even at the single-cell level (9, 10), downregulation of mRNA marker in tumor cells, or low-level transcription of the selected target in the hematopoietic compartment could compromise both the sensitivity and the specificity of molecular methods (11-13). The selection of novel gastrointestinal cancer–specific transcripts and the development of quantitative RT-PCR assays remain outstanding research questions (14, 15).

Our study aims to identify novel gastrointestinal cancer–specific mRNA markers for detection of CTC in the blood. The development of mRNA biomarker assay for CTC detection was conducted following the proposed guidelines of the Early Detection Research Network from the National Cancer Institute (16). The phase I preclinical study was done by means of bioinformatics tools to obtain a quantitative, comprehensive, and unbiased profile of gene expression of colorectal, gastric, and pancreatic adenocarcinoma. These *in silico* data were used to identify and prioritize molecular markers that are highly expressed in gastrointestinal cancers but absent in hematopoietic-derived libraries. Selected genes, including *plakophilin-3* (*PKP3*), were evaluated in cell lines and clinical specimens. Finally, a quantitative RT-PCR (qRT-PCR) assay for *PKP3* was developed to identify blood-borne cells in cancer patients (17).

PKP3 is a member of the p120ctn/plakophilin subfamily of β-catenin (*CTNNB1*) and armadillo (*ARM*) proteins. *PKP3* is present in the desmosomes of epithelial cells and binds desmogleins, desmocollin, plakoglobin, desmoplakin, and keratin 18. *PKP3* is involved in desmosome-dependent cell adhesion and is specific to epithelial cells. It also functions in signal transduction (18-21). Little is known about the possible biological role of *PKP*s in tumor invasion and metastasis (22). In addition, knowledge of the regulation of their expression is limited. Recent studies have shown evidence that the *PKP3* protein in tumor may serve as a useful marker for predicting the clinical outcome of head and neck (23) and non–small cell lung (24) cancers. An oncogenic role for *PKP3* in non–small cell lung cancer has been suggested, as *PKP3* promotes growth and invasion in experimental models (24). Other investigators (25) have shown that this protein is highly expressed in various types of adenocarcinomas, including colorectal, pancreatic, and prostate tumors. Recently, *PKP1*, another member of plakophilins, has been described as a marker for subclinical disease in blood and bone marrow in the Ewing family of tumors (26).

To assess *PKP3* as a new surrogate biomarker of CTC, we examined *PKP3* mRNA in the blood of gastrointestinal cancer patients using qRT-PCR. Our findings indicate that quantitative assessment of *PKP3* mRNA can be a marker of subclinical disease in gastrointestinal cancer patients and has potential clinical relevance as a predictor of disease outcome.

Materials and Methods

Patients

Consecutive patients with gastrointestinal cancer from the Medical Oncology Unit at University Hospital in La Coruña (Spain) were included in the study. Inclusion criteria were as follows: patients with a confirmed pathologic diagnosis of invasive adenocarcinoma of the gastrointestinal tract, including colorectal, gastric, and pancreatic tumors; stage I-III patients with no prior systemic therapy for gastrointestinal cancer; stage IV patients without previous systemic therapy or with confirmed cancer progression after such treatment; and patients providing written informed consent. Exclusion criteria were defined as follows: patients with any other previous malignancy; patients with coagulation disorders; patients with platelet count less than 20×10^9 per liter; and patients with any previous systemic therapy for cancer except stage IV patients with progressive disease confirmed at the time of blood sampling.

The diagnostic work-up included a clinical examination, blood sampling with CA 19.9 and carcinoembryonic antigen (CEA) serum determination, an endoscopy (when clinically indicated), a chest X-ray, and computed tomography scanning of the abdomen and pelvis. A chest computed tomography was done on the upper digestive tract and on rectal tumors and stage IV patients. Patients were followed to observe disease progression with imaging every 6 to 12 weeks.

Serum CEA (with an upper limit of normal of 5 ng/mL) and CA 19.9 (with an upper limit of normal of 37 units/mL) levels were determined using enzyme immunoassay test kits (Advia Centaur, Siemens Healthcare Diagnostics), according to the manufacturer's instructions.

Blood samples for qRT-PCR analysis were obtained after surgery, before neoadjuvant chemotherapy, or in the presence of active, clinically and radiologically advanced progressive disease. At least the first 5 mL of blood obtained was discarded to avoid contamination with epidermal cells.

Controls were recruited from the patient families. We only excluded those with a previous history of malignant disease. Thus, controls with different chronic but stable disease (i.e., hypertension, diabetes mellitus, or heart disease, among others) were eligible and consecutively recruited. Controls were selected to include a sex and age distribution comparable to the patient group.

This study was approved by the institutional review board of the Ethic Committee of Clinical Investigation of Galicia (Spain), and written informed consent was obtained from all patients and controls.

Pathologic analysis

Primary tumors and regional lymph nodes collected during surgery were processed on a routine diagnostic basis. Histologic tumor type, depth of tumor invasion, and nodal involvement were analyzed, and the disease was staged and graded according to the tumor-node-metastasis system (27). Residual disease status at the time of blood sampling was classified as R0 when no residual disease was present after surgery, R1 when microscopic residual disease was found, and R2 in the presence of macroscopic disease. Patients from whom blood was obtained before the start of neoadjuvant systemic treatment were categorized as R2.

Processing of blood samples and mRNA isolation

Peripheral venous bloods (10 mL) were collected in EDTA-containing tubes. Samples were stabilized within 1 hour after withdrawal in guanidinium-based RNA/DNA stabilization reagent for blood and bone marrow (Roche) at 10% (v/v) without cell and plasma separation. Isolation reagent for blood and bone marrow (Roche) was used for mRNA extraction according to the manufacturer's protocol with minor modifications (17). Purified poly(A)+ RNA was further processed in qRT-PCR or stored at −80°C until use. The RNA concentration was determined by UV absorption at 260 nm. The A_{260}/A_{280} ratio was calculated to assess RNA quality and purity. In addition, RNA integrity was confirmed by 2% agarose gel electrophoresis and ethidium bromide staining.

Reverse transcription and quantitative real-time PCR

Reverse transcription was done on 0.02 μg of mRNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as described previously (17). Real-time PCR analysis was done with the primers and conditions previously described (17) using a LightCycler 480 (Roche) and SYBR Green I Master (Roche).

The PCR reaction consisted of 10 μL of Master Mix 2× concentration, 1.4 μL of forward (*PKP3* 2F) and reverse (*PKP3* 1R) primers at 5 μmol/L, 4 μL of cDNA, and PCR-grade water up to a final volume of 20 μL in each well in a LightCycler 480 Multiwell Plate 96.

The maximum number of cycles was 50. If no fluorescent signal was detected on the amplification plots after 40 cycles, the marker mRNA was assumed to be absent from the blood samples.

Hypoxanthine-guanine phosphoribosyl-transferase 1 (*HPRT1*) was selected as a housekeeping gene using the human endogenous control gene panel (TATAA Biocenter) as previously reported (17). *HPRT1* amplification served as an internal control and was used to verify the integrity of the RNA and the efficacy of reverse transcription. Any specimen with inadequate *HPRT1* mRNA was excluded from the study.

We verified the specificity of PCR by melting curve analysis. Data analysis was done with Light Cycler 480 Relative Quantification software (Roche). Relative levels of expression were calculated by the $2^{-\Delta\Delta Ct}$ method (28). Each assay was done at least in triplicate. The average value of the replicates was used as quantitative value for each sample. Each assay included marker-positive, marker-negative, and reagent with no template controls.

PKP3 expression in colorectal cancer

Eleven pairs of primary colon carcinoma tissues and matched normal colonic mucosa were obtained from patients undergoing surgery and were frozen and stored at −80°C until the extraction of RNA. We microscopically confirmed that the tumor specimens were predominantly (>80%) cancer tissue. RNA isolation was done using the mirVana isolation kit (Applied Biosystems), according to the manufacturer's instructions. cDNA synthesis was done with 1 μg of total RNA using a SuperScript First-Strand Synthesis kit as described (17). *PKP3* mRNA was quantified using SYBR-Green qRT-PCR and normalized using the $2^{-\Delta\Delta Ct}$ method relative to *HPRT1* as previously described. All PCRs were done in triplicate.

Study design and statistical analysis methods

This project was designed as a prospective early-phase, diagnostic case-control study. The primary aim is to estimate the diagnostic accuracy and usefulness of *PKP3* as measured by qRT-PCR in blood as a clinical biomarker (16). Receiver operating characteristic (ROC) curves were constructed by plotting sensitivity (*Y* axis) versus 1−specificity (*X* axis), and the areas under the curves (AUC) were calculated. Secondary aims include the evaluation of *PKP3* mRNA blood levels in gastrointestinal cancer patients and the disease characteristics of these patients. Non normality of the distribution of *PKP3* values was confirmed by Kolmogorov-Smirnov test. Thus, nonparametric tests (Mann-Whitney and Kruskal-Wallis tests) were used to analyze the potential association between *PKP3* expression and the clinical and pathologic features of the study subjects.

Progression-free survival (PFS) was measured as the time between the baseline blood sampling for *PKP3* analysis and the documentation of first tumor progression based on clinical and radiological studies or death. Patients who were alive and progression-free at the time of analysis were censored by using the time between the baseline *PKP3* assessment and their most recent follow-up evaluations. Kaplan-Meier methodology was used to estimate median PFS.

Logistic regression analyses were used to assess the effect of *PKP3* mRNA levels on the risk of disease progression or death. Patients were classified according to their PFS status at 2 years into two groups: disease progression or death and alive without disease progression. Univariate and multivariate analyses were done. Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated.

For all analyses, a *P* value of less than 0.05 was considered statistically significant. SPSS Software (version 16.0) was used for the analysis.

The study design and results are presented in accordance with the REMARK guidelines (29).

Results

Patients and clinical data

From July 2004 to July 2006, 64 consecutive patients with histologically proven gastrointestinal cancer and 23 matched controls were recruited for this study. The clinical characteristics of the cancer subjects are shown in Table 1.

Blood was obtained after R0 or R1 surgery in 17 patients. In 47 patients, blood samples were obtained before neoadjuvant chemotherapy or in the presence of active metastatic disease, both of which were categorized as R2 at the time of blood sampling.

All patients were followed up until death or the end of the study. Tumor progression was detected in 52 patients (81.2%). The median PFS was 37.7 weeks (95% CI, 28.2-47.2). Forty-six patients (71.9%) died of advanced disease. Fifty PFS events had occurred at 2 years of follow-up. The mean follow-up time for the patients still alive at the time of the analysis was 192.2 ± 41.2 weeks (median, 204.6 weeks; range, 124.3-239.4 weeks).

Table 1. Patients and clinicopathologic data

Age (y)		
Median (SD)	62.3 (10.1)	
Range	31-80	
	N	%
<65	36	56
≥65	28	44
Gender		
Female	22	34
Male	42	66
Primary tumor site		
Colon and rectum	48	74
Stomach	8	12.5
Pancreas	8	12.5
Stage		
I-II	5	7.8
III	14	21.9
IV	45	70.3
pT		
pT_1-pT_2	8	14.1
pT_3	37	64.9
pT_4	12	21.1
pT_x	7	9.9
pN		
pN_0	10	15.6
pN_1	29	45.3
pN_2	11	17.2
pN_x	14	21.9
M		
M_0	20	31.2
M_1	44	68.8
R status		
R_0-R_1	17	26.6
R_2	47	73.4
No. of metastatic sites		
0	20	31.2
1	32	50
≥2	12	18.8
Location of metastasis		
None	20	31.2
Liver only	26	40.6
Liver and other	12	18.8
Nonliver metastasis	6	9.4
Grade		
1	10	15.6
2	44	68.8
3	10	15.6
Vascular/perineural invasion		
Unknown	16	25
No	22	34.4
Yes	26	40.6

Expression of PKP3 mRNA transcripts in blood samples

Real-time quantitative assessment of *PKP3* mRNA was done using 87 blood samples (64 patients and 23 controls). *PKP3* transcripts were detected in the blood of 93.8% (60 of 64) of patients but only in 39.1% (9 of 23) of controls ($P < 0.0001$; Fisher's exact test). The mean relative *PKP3* mRNA expression was 48.45 (95% CI, 29.61-67.29) in cancer patients and 2.8 (95% CI, 0.14-5.48) in controls ($P < 0.0001$, *U*-Mann-Whitney test).

ROC curves were constructed by plotting sensitivity versus 1−specificity. Comparing the relative *PKP3* levels in patients and controls, the AUC was 0.852 (95% CI, 0.76-0.94; $P < 0.0001$). Although the values of the AUCs were lower, statistically significant results were obtained when analyzing *PKP3* levels in controls compared with stage I-III patients (AUC, 0.769; 95% CI, 0.623-0.915; $P = 0.003$) and stage I-III patients compared with stage IV patients (AUC: 0.678; 95% CI, 0.526-0.829; $P = 0.026$; Fig. 1).

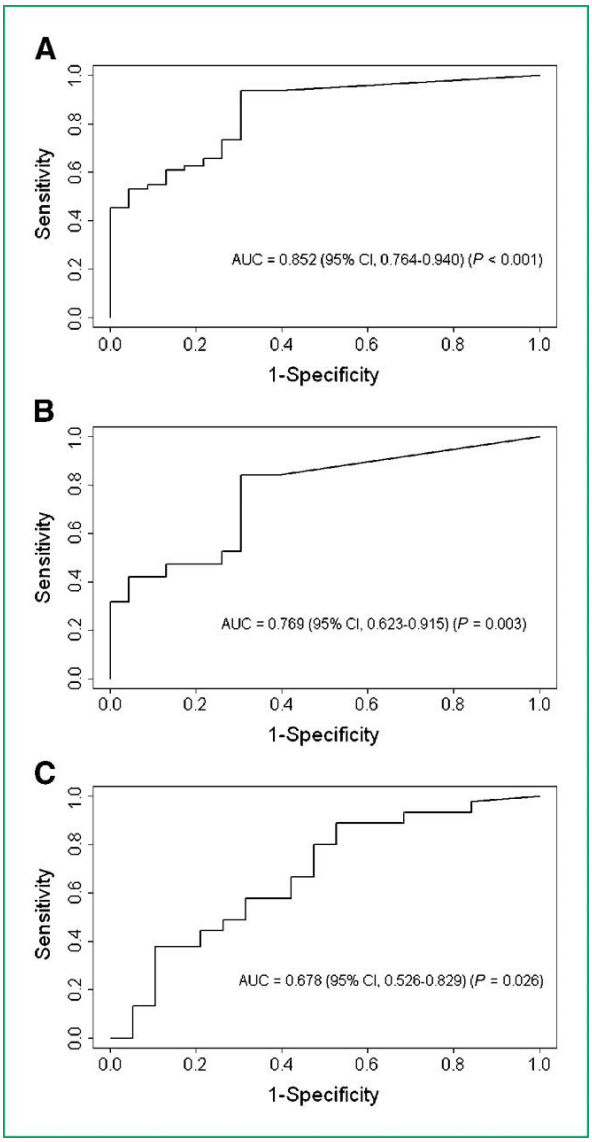

Figure 1. ROC curves were constructed by plotting sensitivity versus 1−specificity. *PKP3* mRNA relative levels were quantified in blood obtained from patients with gastrointestinal cancer (n = 64) and from controls (n = 23). Curves are shown comparing controls versus cancer patients (A), controls versus stage I-III patients (B), and stage I-III patients versus stage IV patients (C). AUCs, 95% CIs, and P values are shown.

In addition, ROC curves were analyzed, comparing the *PKP3* levels in blood in each subgroup of patients (colorectal, gastric, and pancreatic cancer) with the *PKP3* levels in the control group. Statistically significant results were obtained in each pair-wise analysis, and the AUCs were 0.875 (95% CI, 0.789-0.965; $P < 0.001$) for colorectal cancer, 0.821 (95% CI, 0.635-1; $P = 0.008$) for gastric cancer, and 0.745 (95% CI, 0.570-0.963; $P = 0.042$) for pancreatic cancer.

Different cutoff strategies were used to estimate *PKP3* levels as a surrogate for discrimination between the presence and absence of CTC in blood samples from gastrointestinal cancer patients (Table 2). Considering the 95% CI for the mean relative levels in healthy or benign donors, we set the *PKP3* cutoff at 5.5. *PKP3* counts higher than 5.5 were found in 60.9% of cancer patients. The specificity was 82.6%. Using values obtained in the ROC curve analysis, PKP3 levels higher than 15.25 were observed in 53.1% of the cancer patients; the specificity was 95.7%.

Table 2. Diagnostic performance of *PKP3* mRNA qRT-PCR

	Sensitivity (%; 95% CI)	Specificity (%; 95% CI)	Positive predictive value (%; 95% CI)	Negative predictive value (%; 95% CI)	Accuracy (%; 95% CI)
Patients and controls					
PKP3 >5.5	60.9 (47.9-72.6)	82.6 (60.5-94.3)	90.7 (76.9-96.9)	43.2 (28.7-58.9)	66.7 (55.7-76.2)
PKP3 >15.25	53.1 (40.3-65.6)	95.7 (40.3-65.6)	97.1 (83.4-99.9)	42.3 (29.0-56.7)	64.4 (53.3-74.1)
Stage I-III and stage IV patients					
PKP3 >5.5	66.67 (50,9-79,6)	52.6 (29.5-74.8)	76.92 (60.3-88.3)	40.0 (21.8-61.1)	62.5 (49.5-74.0)
PKP3 >15.25	57.8 (42.2-72.0)	57.9 (33.9-78.9)	76.5 (58.4-88-6)	36.7 (20.5-56.1)	57.81 (44.9-69.8)

Clinical significance of PKP3 mRNA in blood

The clinical and pathologic characteristics and the *PKP3* mRNA expression in blood from cancer patients are shown in Table 3. A higher relative level of blood expression of *PKP3* was associated with a more advanced stage ($P = 0.025$), pT_{3-4} ($P = 0.028$), metastatic disease ($P = 0.021$), and residual disease (R2) status ($P = 0.037$).

The relative expression of *PKP3* mRNA in the blood was higher in patients with colorectal (mean, 51.5) and pancreatic (mean, 53.3) cancer than in gastric cancer (mean, 25.4) patients, although these differences were not significant (Kruskal-Wallis test, $P = 0.702$).

In pair-wise comparisons of the mean relative *PKP3* mRNA expression in blood in each subgroup of patients (colorectal, gastric, and pancreatic cancer) with the expression in control group, the relative expression of *PKP3* in each cancer subgroup was higher than in controls (*U*-Mann-Whitney test; $P = 0.0001$, $P = 0.006$, and $P = 0.043$ for colorectal, gastric, and pancreatic cancer, respectively).

CEA and CA 19.9 serum levels were increased above the upper limits of normal in 46.8% and 41.3% of the patients, respectively. There were no correlations between *PKP3* mRNA levels and either CEA or CA 19.9 in serum (Spearman's ρ coefficients = 0.144 and 0.010, respectively).

To explore the possible influence of recent surgery on the circulation of tumor cells, we analyzed *PKP3* levels according to the time interval from operation and blood sampling (Table 4). The mean time from surgery to blood sampling for *PKP3* mRNA quantification was 354.3 ± 396.3 days (median, 126.5 days; range, 30-1,392 days). The 25th percentile was 48 days. There was no significant difference in *PKP3* levels between time intervals (<48 or ≥48 days) from the last surgery.

Table 3. Distribution of clinicopathologic parameters and levels of *PKP3* mRNA in the blood

Parameter	*n*	*PKP3* (mean)	SEM	*P*
Age (y)				0.656
<65	35	52.3	16.2	
≥65	29	40.4	13.5	
Gender				0.354
Male	42	47	9.6	
Female	22	51.2	20.9	
Primary tumor site				0.702*
Colon and rectum	48	51.5	11.6	
Stomach	8	25.4	10.6	
Pancreas	8	53.3	27.2	
Stage				***0.025***
I-III	19	44.6	25.9	
IV	45	48.5	10.5	
pT				***0.028***
pT_1-T_2	8	29.4	27.4	
pT_3-T_4	49	50	11.9	
pN				0.128
Node negative	10	66.7	21.5	
Node positive	42	42.6	12.5	
M				***0.021***
M_0	20	38.2	22.2	
M_1	44	53.1	9.4	
R status				***0.037***
R_0-R_1	17	21.6	7.8	
R_2	47	58.2	12.3	
No. of metastatic sites				0.053*
0	20	38.2	22.2	
1	32	52.2	11.4	
≥2	12	55.7	17.3	
Location of metastasis				0.069
None	20	38.2	22.2	
Liver	38	54	10.7	
Nonliver metastasis	6	47.5	15.8	
Grade				0.383*
1	10	32.5	20.5	
2	44	56.2	12.6	
3	10	30.5	11.7	
Vascular/perineural invasion				0.808
No	23	53.9	20.1	
Yes	27	41.5	11.2	

NOTE: *PKP3* mRNA relative expression levels are shown as arbitrary units. Mann-Whitney Test or *Kruskal-Wallis Test were used. *P* values of less than 0.05 are italicized.

Table 4. *PKP3* levels according to time interval between surgery and blood sampling

	n	*PKP3* median (SD)	P^*
Patients with previous surgery	50	39.56 (55.96)	
Time from surgery to blood sampling <48 d	11	46.29 (67.01)	0.897
Time from surgery to blood sampling ≥48 d	39	37.65 (53.29)	

↵*Two-sided Mann-Whitney test.

Logistic regression was used to predict PFS event at 2 years based on *PKP3* mRNA levels in the blood of cancer patients (Table 5). In univariate analysis, using a *PKP3* cutoff value of 5.5 as previously defined, higher *PKP3* levels were associated with a higher risk of cancer progression or death (OR, 3.875; 95% CI, 1.781-8.430; $P = 0.001$).

Table 5. Logistic regression model for predictive factors associated with PFS events

Variable	Subset	Univariate		Multivariate	
		OR (95% CI)	*P*	OR (95% CI)	*P*
PKP3 mRNA	≤5.5/>5.5	3.875 (1.781-8.430)	0.001	0.898 (0.104-7.777)	0.922
Residual disease	R0-1/R2	2.898 (1.823-4.608)	<0.0001	1.797 (0.414-7.798)	0.434
Stage	I-II-III/IV	2.699 (1.791-4.067)	<0.0001	17.32 (1.150-260.8)	0.039
Vascular/perineural invasion	No/yes	5.75 (1.989-16.626)	0.001	14.885 (1.256-176.4)	0.032
Lymph node	Negative/positive	2.818 (1.417-5.607)	0.003	0.223 (0.018-2.775)	0.244
Invasion depth (pT)	pT_{1-2}/pT_{3-4}	1.901 (1.365-2.646)	<0.0001	0.317 (0.034-2.946)	0.313
Age	<65 y/≥65 y	2.500 (1.201-5.205)	0.014	0.093 (0.008-1.026)	0.053

Considering *PKP3* mRNA levels as a continuous variable (30) in the logistic regression analysis, increasing levels were associated with the risk of cancer progression or death (OR, 1.011; 95% CI, 1.001-1.020; $P = 0.025$).

Using multivariate logistic regression analysis, only stage IV (OR, 17.32; 95% CI, 1.150-260.8; $P = 0.039$) and vascular/perineural invasion (OR, 14.885; 95% CI, 1.256-176.4; $P = 0.032$) remained significant for PFS events.

PKP3 is overexpressed in colorectal carcinomas

PKP3 mRNA content was determined in 11 pairs of frozen colorectal tumors and matched normal colon epithelia using real-time qRT-PCR. The *PKP3* mRNA levels were significantly higher in cancer tissue than in normal colonic mucosa ($P = 0.001$). Ten cancer samples (90.9%) overexpressed *PKP3* in comparison with the normal tissues (mean fold increase, 3.47 ± 2.25).

Discussion

PCR amplification of tissue- or tumor-specific mRNA is a powerful analytic tool for surrogate detection of CTC. Real-time RT-PCR allows for quantification of the cancer cell load in the peripheral blood, and, theoretically, the determination of cutoff values indicating mRNA expression levels of clinical relevance in cancer patients. However, several limitations of this approach have been described. Both sensitivity and specificity are dependent on the expression level of markers in tumor cells as well as their background expression in the blood. Thus, the identification of appropriate target mRNA transcripts that are useful for clinical detection of CTC in the blood remains clearly a remarkable issue.

In a previous study (17), we used DDD (Digital Differential Display) to search SAGE (Serial Analysis of Gene Expression) and EST (Expressed Sequence Tags) libraries of the Cancer Genome Anatomy Project, and we constructed a gene expression profile of colorectal, gastric, and pancreatic cancers in comparison with the hematopoietic gene profile. *PKP3* was selected, taking into account its upregulation in gastrointestinal cancer and its low-level expression in the hematopoietic compartment. In addition, the diagnostic performance of the *PKP3* mRNA assay was better when comparing with the results obtained with an *EGFR* mRNA assay in the same blood sample cohort based on the ROC curve analysis (17).

Building on our phase I preclinical study, the current study was intended to assess the diagnostic performance of quantitative RT-PCR detection of mRNA *PKP3* in the blood of patients with different gastrointestinal cancers, including colorectal, pancreatic, and gastric adenocarcinomas. Patients were included to accurately reflect everyday clinical practice. Our results showed that blood mRNA *PKP3* levels were significantly correlated with different pathologic and clinical prognostic factors in

gastrointestinal cancer, including stage, depth of tumor invasion, presence of metastases, and residual disease status.

The sensitive quantification of *PKP3* and the definition of cutoff values improved the specificity of our assay. Different cutoff strategies were used to discriminate between the presence and absence of CTC in blood samples from gastrointestinal cancer patients. Threshold levels estimated with the ROC curves were associated with the risk of relevant clinical end point as PFS events by logistic regression analysis. Furthermore, the *PKP3* mRNA levels, which were analyzed as a continuous variable, were associated with the risk of cancer progression and death.

Previous studies have shown that molecular markers for CTC in gastrointestinal cancer patients are increasingly detected when blood is obtained perioperatively or intraoperatively (31-33). However, the prognostic impact of this tumor cell shedding during surgery has yet to be shown. In our study, blood samples were obtained several weeks after surgery if an operation was done. To explore the possible influence of recent surgery on the circulation of tumor cells, *PKP3* levels were analyzed according to time interval between surgery and blood sampling; however, no significant differences in *PKP3* levels between time intervals were found. Thus, we hypothesized that only circulating and viable cancer cells resistant to anoikis were detected using the mRNA *PKP3* assay.

In a previous study, Furukawa et al. (24) had shown that overexpression of *PKP3* in non–small cell cancer cell lines promotes cell motility and invasion by association with dynamin 1–like protein. Moreover, cancer cells mainly exhibited a cytoplasmic distribution of overexpressed PKP3 protein, consistent with the results of a previous report (23). Semiquantitative RT-PCR analysis had revealed (24) that there was no significant correlation of gene expression between *PKP3* and *PKP3*-associated desmosomal components in lung cancer samples and stable transfected cells.

For epithelial malignancies, the epithelial-mesenchymal transition (EMT) is considered one of the crucial events in the metastatic process (2, 34). EMT involves the disruption of epithelial cell homeostasis and the acquisition of a migratory mesenchymal phenotype, allowing these cells to invade and circulate in the blood to the site of metastasis development. Interestingly, Aigner et al. (35) have shown that the transcriptional deregulation of *PKP3* by the E-cadherin repressor *ZEB1* contributes to disintegration of intercellular adhesion and to the EMT. At the invasive front of tumors, where EMT and releasing of CTCs take place, *ZEB1* is upregulated and thus *PKP3* protein associated with desmosomes are downregulated (24, 35), promoting desmosome instability and increased cell migration. However, the cancer cells still display a *PKP3* cytoplasmic pool (35). This cytoplasmic pool may be in relation with additional functions for *PKP3* in cells, beyond its participation in desmosomes. During environmental stress conditions, like those occurring during cancer progression, *PKP3* is found associated with other proteins in “stress granules” containing stalled translation initiation complexes (36). This indicates that *PKP3* is involved in RNA metabolism, protein translation, and stress response (36). These findings could explain the *PKP3* expression in CTCs after undergoing EMT. An alternative or complementary explanation is that CTCs preserve epithelial components, like *PKP3*, waiting for reversing their mesenchymal status through mesenchymal-to-epithelial transition and generate metastasis foci. In line with this, the majority of CTCs isolated in breast cancer patients by the presence of epithelial surface markers (*Ep-CAM*) also show coexpression of EMT and stem cell markers (37). In addition, we have detected *PKP3* mRNA expression also in colorectal cancer metastasis (17).

The limitations of this study must be considered. Patients with adenocarcinomas from different primary digestive sites (including colorectal, gastric, and pancreatic tumors) were included, which may influence the clinical relevance of *PKP3* quantification to any one group. However, our results suggest that *PKP3* can be considered an epithelial-selective marker and not an organ-specific marker. Bioinformatics analysis has shown high expression levels in different normal epithelia and tumors, including gastric, colorectal, and pancreatic carcinomas (17). Using real-time quantitative PCR, we showed that *PKP3* mRNA was overexpressed in colorectal tumors in comparison with matched normal colonic tissues. Although the inclusion of patients with different stages of disease and residual tumor situations could be considered an additional limitation of this study, we suggest that this pragmatic design accurately reflects the patients attending everyday in the oncology clinic. Thus, the diagnostic performance of *PKP3* mRNA quantification has been estimated in a cohort of patients truly representative of those found in the clinical setting. However, to adequately assess the prognostic role, if any, of *PKP3* mRNA levels in the blood, a larger, more homogeneous cohort of patients is clearly needed.

In conclusion, our findings indicate that the quantitative assessment of *PKP3* mRNA in blood can be used as a marker of subclinical disease and, thus, holds potential clinical relevance as a predictor for disease outcome.

Disclosure of Potential Conflicts of Interest
The authors indicated no potential conflicts of interest.

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