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Multimarker Gene Analysis of Circulating Tumor Cells in Pancreatic Cancer Patients: A Feasibility Study

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Key Words
Circulating tumor cells · Pancreatic cancer · Reverse transcriptase real-time polymerase chain reaction

Abstract
Objective: The aim of this study was to develop an immunomagnetic/real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay and assess its clinical value for the molecular detection of circulating tumor cells (CTCs) in peripheral blood of pancreatic cancer patients. ***Methods:*** The presence of CTCs was evaluated in 34 pancreatic cancer patients before systemic therapy and in 40 healthy controls, through immunomagnetic enrichment, using the antibodies BM7 and VU1D9 [targeting mucin 1 and epithelial cell adhesion molecule (EpCAM), respectively], followed by real-time RT-PCR analysis of the genes *KRT19*, *MUC1*, *EPCAM*, *CEACAM5* and *BIRC5*. ***Results:*** The developed assay showed high specificity, as none of the healthy controls were found to be positive for the multimarker gene panel. CTCs were detected in 47.1% of the pancreatic cancer patients before the beginning of systemic treatment. Shorter median progression-free survival (PFS) was observed for patients who had at least one detectable tumor-associated transcript, compared with patients who were CTC negative. Median PFS time was 66.0 days [95% confidence interval (CI) 44.8–87.2] for patients with baseline CTC positivity and 138.0 days (95% CI 124.1–151.9) for CTC-negative patients (p = 0.01, log-rank test). ***Conclusion:*** Our results suggest that in addition to the current prognostic methods, CTC analysis represents a potential complementary tool for prediction of outcome in pancreatic cancer patients. Copyright © 2012 S. Karger AG, Basel

Introduction

Pancreatic cancer is the 10th most commonly diagnosed cancer and has the highest mortality rate, as 96% of the patients diagnosed with this malignancy die within the first 5 years [1]. Pancreatic cancer lacks early symptoms and is, as a consequence, often diagnosed at a late stage when disease has spread to neighboring tissues, beyond the treatment window of curative resection [2]. The inaccessibility of the pancreas further limits the possibility of surgical removal, and the aggressive nature of the tumor leads to a rapid progression that is strongly resistant to chemotherapy [3]. Moreover, the conventional prognostic indicators to predict patient outcome are often imperfect, owing mainly to tumor plasticity and sub-

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jective assessment criteria [4]. Therefore, there is an urgent need for the establishment of new sensitive prognostic methods capable of identifying patients with a worse prognosis or those destined to progress quickly.

During recent years, the analysis of circulating tumor cells (CTCs) has become a promising diagnostic tool in oncology. However, although the prognostic value of CTCs has been extensively studied in breast, colorectal and prostate cancer [5–11], its role in pancreatic cancer is still poorly investigated. To date, only a few studies have assessed CTCs in peripheral blood (PB) of pancreatic cancer patients, with most of the published work reporting small cohorts, low CTC positivity rates and contradictory results [12].

In the present study, we have employed immunomagnetic enrichment and real-time reverse transcriptase polymerase chain reaction (RT-PCR) as the methodological basis for the development of a CTC detection method. Our goal was to create an accurate assay that would improve the detection of CTCs in PB of pancreatic cancer patients and, at the same time, avoid false-positive results. Moreover, we aimed to evaluate whether CTC detection could be used as a tool to refine prognosis in pancreatic cancer management.

Patients and Methods

Patient Selection and PB Sampling

Written informed consent was obtained from all participants, and the study was approved by the local medical ethical committee. The principal inclusion criteria were as follows: patients with histologically and radiographically proven pancreatic adenocarcinoma, initiating any first- or second-line systemic therapy. Patients with a history of previous malignancy and patients with active infection were excluded.

Whole blood samples (10.0 ml) were collected into EDTA tubes from all subjects before therapy (Sarstedt AG & Co., Nümbrecht, Germany), and CTCs were isolated within 4 h of specimen collection. A group of healthy, anonymous control subjects, who were randomly selected from hospital staff, were asked to participate in the study, and blood sampling was performed as described above.

Patient Follow-Up

Patients underwent chemotherapy as appropriate for their diagnosis and disease evaluation by their medical oncologist according to the institutional guidelines. The evaluation included a physical examination, a complete blood count, blood chemical tests, screening for serum tumor markers, radiography, computed tomographic scan and magnetic resonance imaging according to tumor type and stage. The planned reevaluation for patients with metastatic disease was performed every 3 months. Response was evaluated according to the clinical criteria codified by the Response Evaluation Criteria in Solid Tumors [13] by a team of medical oncologists and radiologists. Each disease assessment was classified as a complete response, partial response, stable disease or progressive disease. The primary end point for metastatic patients was time to progression. For response to therapy in the metastatic setting, the favorable group was defined as having nonprogressive disease (complete response, partial response and stable disease categories), and the unfavorable group was defined as those patients who suffered progressive disease or death.

CTC Isolation from Blood Samples

CTCs were isolated from PB using 200 μl of BM7/VU1D9 antibodies coupled directly to immunomagnetic 4-μm Dynabeads® (Invitrogen, Karlsruhe, Germany). Beads were incubated with the PB on a low-speed rotating device for 20 min at room temperature, after which labeled cells were separated using an external magnetic particle concentrator. The bead fraction was washed 5 times with phosphate-buffered salt solution, and the retained mucin 1-positive and/or epithelial cell adhesion molecule (EpCAM)-positive cells were lysed in 400 μl of Tris-HCl lysis buffer (included in the Dynabeads mRNA Direct™ Kit, Invitrogen) and stored at –85°C until mRNA isolation and cDNA synthesis.

mRNA Isolation and cDNA Synthesis

mRNA isolation from the lysed enriched cells was performed with the Dynabeads mRNA Direct Kit according to the manufacturer's instructions. Sensiscript® Reverse Transcriptase (Qiagen, Hilden, Germany), recommended for first-strand cDNA synthesis using <50 ng RNA, was used for reverse transcription of the isolated and purified mRNA in combination with the Dynabeads oligo$(dT)_{25}$ (Invitrogen), according to the manufacturer's guidelines. Reverse transcription was performed in 0.5 μl of RNasin® Ribonuclease Inhibitor (40 U/μl; Promega, Mannheim, Germany), 4 μl of reverse transcriptase buffer, 4 μl of dNTPs (5 mM each) and 2 μl of Sensiscript Reverse Transcriptase and synthesized in a thermocycler under the following conditions: 60 min at 37°C followed by 5 min at 93°C. The resulting 40 μl of cDNA was stored at –20°C until further use.

Multimarker Real-Time PCR Analysis

Reverse transcription resulted in cDNA which was the template for tumor cell detection and characterization by real-time PCR. The analysis of tumor-associated mRNA isolated from CTCs was performed for 5 tumor-associated transcripts: *KRT19*, *MUC1*, *EPCAM*, *CEACAM5* and *BIRC5*. Primers were selected using the Universal ProbeLibrary® system, and their sequences can be seen in table 1. The selected primers were designed to be intron spanning (exon specific) so as to eliminate reactivity with genomic DNA. The amplification of *ACTB* (primers: forward, 5′-GAAGAGCCAAGGACAGGTAC-3′; reverse, 5′-CAACTTCATCCACGTTCACC-3′) served as a reference internal control and was used to verify the integrity of the RNA and the quality of the samples. PCR amplifications were performed on the Rotor-Gene 3000 in a total volume of 25 μl. Each reaction contained 12.5 μl of the reaction buffer MESA FAST qPCR MasterMix Plus for SYBR® assay (Eurogentec, Cologne, Germany), including dNTPs (together with dUTP), Meteor*Taq* DNA polymerase, $MgCl_2$ (4 mM final concentration), SYBR Green I and stabilizers, 0.1 μl of each primer (100 pmol/μl), 2 μl of cDNA and 10.3 μl of RNase-free H_2O. The thermal profile used for real-time PCR was as follows: after a 5-min denaturation at 95°C, 40 cycles were carried

Table 1. Intron-spanning primer pairs for each selected gene

Marker	NM reference	Primer sequence		Location	Product size, bp
KRT19	NM_002276.2	forward reverse	GCCACTACTACACGACCATCC CAAACTTGGTTCGGAAGTCAT	525–545, exon 1 650–630, exon 2/3	126
MUC1	NM_002456.4	forward reverse	TCGTAGCCCCTATGAGAAGG CCACTGCTGGGTTTGTGTAA	795–814, exon 7/8 865–846, exon 8	71
EPCAM	NM_002354.2	forward reverse	CGTCAATGCCAGTGTACTTCA TTTCTGCCTTCATCACCAAA	448–508, exon 2 575–553, exon 3	88
CEACAM5	NM_004363.2	forward reverse	ACCACAGTCACGACGATCAC CTCCACGGGGTTGGAGTT	1052–1071, exon 4 1129–1112, exon 5	78
BIRC5	NM_001168.2	forward reverse	GCCCAGTGTTTCTTCTGCTT CCGGACGAATGCTTTTTATG	284–303, exon 2 369–350, exon 3	86

out with denaturation at 95°C for 5 s, annealing at 59°C for 20 s and extension at 72°C for 12 s.

PCR efficiency, linearity and sensitivity for each gene were validated with a standard curve constructed from a simultaneously run serially diluted cDNA pool of human PB lymphocytes (PBLs) and the tumor cell line ZE, which expresses all the gene markers analyzed in this assay. Negative controls included samples without reverse transcriptase and samples in which cDNA was replaced with genomic DNA. All values were obtained from the quantitative cycle (Cq) at which the increase in SYBR green fluorescent signal associated with an exponential increase in PCR products reached the fixed threshold value of 0.25.

Random PCRs were analyzed by gel electrophoresis in order to determine the specificity of the assay and ensure that with the PCR conditions and the different primer sets used, the product of the expected size was amplified.

Data Analysis

Taking into consideration all the limitations presented by relative and absolute quantification strategies, we used real-time RT-PCR to detect the presence of a gene rather than to quantify it accurately. This approach is referred to as qualitative RT-PCR. However, in order to obtain an accurate yes/no answer, information about the analytical sensitivity of the real-time RT-PCR assay must be determined [14]. In the present study, we adopted the approach proposed by Caraguel et al. [15]. According to these authors, the analytical sensitivity of PCR, also referred to as the lower limit of detection, is defined as the minimum concentration of analyte detected at which 50% of the tested samples are positive and fit within the linear dynamic range of the reaction. Therefore, to demonstrate that the assay detects at least 50% of samples at a specified concentration with 95% confidence, a standard curve needs to be constructed and 5 replicates per dilution must be tested. The analytical sensitivity is then estimated as the last serial linear concentration that yielded positives in all 5 replicates, meaning that the 95% confidence interval (CI) for the probability of testing positive is 47.8–100%. The corresponding Cq value obtained as the lower limit of detection for each gene is selected as the analytical cutoff. Any Cq value above this defined limit is considered not reliable. In the present study, serial 10-fold dilutions of a cDNA pool of human PBLs and ZE tumor cells were used to construct the standard curves.

Statistical Analysis

The linearity, efficiency and analytical sensitivity of the real-time RT-PCR assay were assessed by linear regression analysis. Standard curves were constructed for each marker, and from the equation obtained after regression analysis, the following parameters were determined: sensitivity, given by the y intercept; linearity, expressed as the correlation coefficient (R^2), and efficiency of the assay, determined by the slope of the log-linear phase of the amplification reaction.

The clinical utility of CTC detection in pancreatic cancer patients was assessed by means of survival analysis. Progression-free survival (PFS) was defined as the time between the baseline CTC assessment (the initiation of treatment) and the documentation of first radiographic disease progression or death. Patients who were alive and progression free at the time of analysis were censored using the time between the baseline CTC assessment and their most recent follow-up evaluations. PFS in CTC-positive and CTC-negative groups was compared with the Kaplan-Meier method, and differences were tested with the log-rank test. Potential correlations between CTC findings and the clinicopathological characteristics of the patients were tested using either a χ^2 test or Fisher's exact test.

A p value of <0.05 was considered to be statistically significant. All analyses were carried out using SPSS (version 17.0, SPSS, Chicago, Ill., USA).

Results

Validation of the Multimarker Panel for CTC Detection

The specificity of the real-time RT-PCR products was analyzed by high-resolution gel electrophoresis. Sharply defined bands of the correct size (76 bp for *ACTB*, 126 bp for *KRT19*, 71 bp for *MUC1*, 88 bp for *EPCAM*, 78 bp for

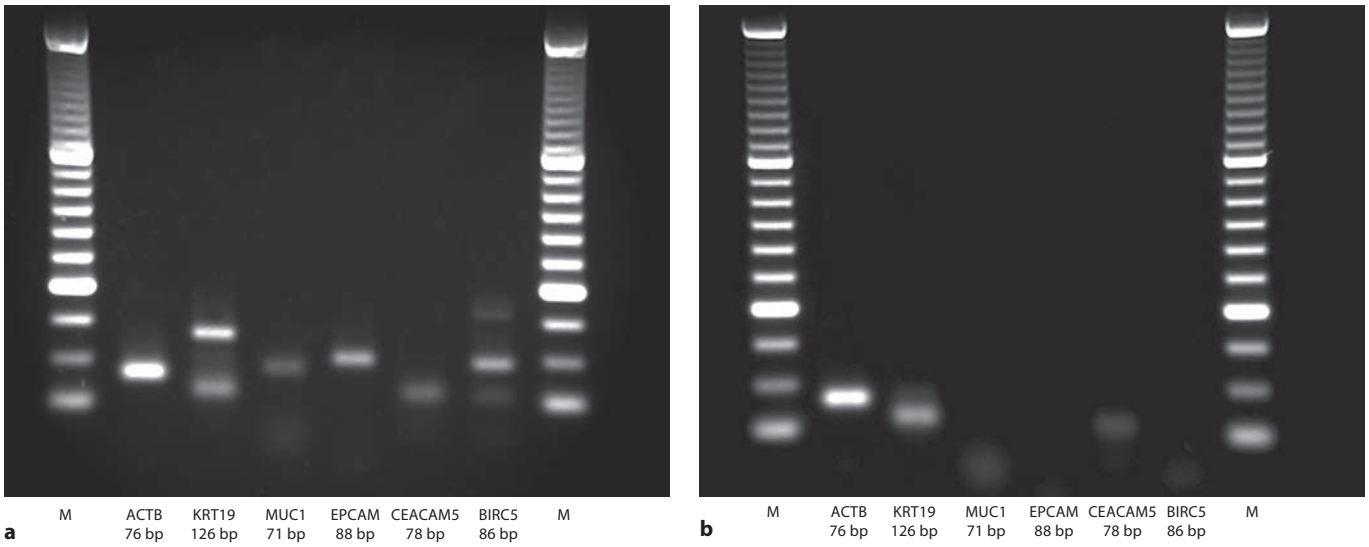


Fig. 1. Electrophoresis gel analysis of representative RT-PCR results for a CTC-positive pancreatic cancer patient (**a**) and a CTC-negative pancreatic cancer patient (**b**). Lane M: 50-bp ladder DNA size marker (Fermentas, St. Leon-Rot, Germany).

CEACAM5 and 86 bp for *BIRC5*) were obtained for the PCR products of the analyzed genes (fig. 1), and primer dimers were found to be present in cDNA samples tested for *KRT19* and *BIRC5*. Bands visible after electrophoresis were concordant with the real-time RT-PCR results obtained.

To determine the linearity, efficiency and analytical sensitivity of the multimarker real-time RT-PCR assay, we analyzed serial 10-fold dilutions of a cDNA pool of human PBLs and ZE tumor cells in 5 different experiments for the 5 tumor-associated transcripts *KRT19*, *MUC1*, *EPCAM*, *CEACAM5* and *BIRC5*. Calibration curves from these data showed linearity over the entire quantification range (1–10^4 tumor cells) and $R^2 \geq 0.99$ in all of the cases, indicating a precise log-linear relationship. The PCR efficiency for the multimarker panel, expressed as $E = 10^{-1/slope}$, ranged from 96 to 104% (data not shown). The analytical sensitivity was estimated as the last serial linear concentration that yielded positives in all 5 replicates, and the corresponding Cq value was selected as the analytical cutoff. According to the results obtained, the analytical Cq cutoff, under which a sample was considered to be positive for the corresponding marker gene, was 36.0 for *KRT19,* 37.1 for *MUC1,* 36.0 for *EPCAM,* 37.8 for *CEACAM5* and 35.0 for *BIRC5*.

Sample Quality

The assessment of sample quality and RNA integrity are critical steps in obtaining meaningful gene expression data. Working with low-quality RNA may strongly compromise the acquisition of reliable results. Therefore, experiments were performed in order to establish a threshold criterion that delineates the sample quality and integrity sufficient to yield reliable results.

Random samples were tested for the leukocyte marker CD45. Results revealed that in all the samples with an *ACTB* Cq value >30.0, no CD45 amplification was found. This indicates that at *ACTB* Cq values higher than 30.0, the RNA integrity is fully compromised. Therefore, a total of 6 healthy blood donor samples and 2 patient samples were excluded from this study because their *ACTB* Cq value was >30.0. Moreover, in order to evaluate false-positive marker expression due to unspecific background levels of mononucleated cells, samples containing different concentrations of lymphocytes were tested for the multimarker panel. Real-time RT-PCR analysis of samples containing 10^3 PBLs presented a mean *ACTB* Cq value of 26.2 (range 24.1–27.4) and showed no amplification of any of the marker genes used in the present study. On the other hand, in samples containing 10^4 PBLs, unspecific amplification of the markers *MUC1, EPCAM* and *BIRC5* was found. These samples presented *ACTB* Cq values ranging from 21.1 to 24.0. Therefore, in order to avoid

false-positive results due to lymphocyte contamination, a total of 3 patient samples with *ACTB* Cq values below 24.0 were excluded from this study.

Patient Characteristics

Between April 2009 and June 2011, a total of 40 patients were enrolled, 34 of whom met the inclusion and exclusion criteria [5 patients had to be excluded due to poor sample quality (inadequate *ACTB* Cq value) and 1 patient was excluded due to a history of previous malignant disease]. During the median follow-up period of 380 days (range 61–789), evidence of disease progression was documented in 23 patients and death had occurred in 9 patients. Detailed clinicopathological characteristics of the patients are given in table 2.

CTCs at Baseline

At baseline, 47.1% of patients showed amplification of at least one tumor-associated marker mRNA in their PB sample. Positivity rates for each individual marker were as follows: 20.6% for *KRT19* and *MUC1*, 23.5% for *EPCAM*, 2.9% for *CEACAM5* and 17.6% for *BIRC5*. A total of 20.6% of the patients were positive for more than one marker. No amplification of the marker genes was seen in the 40 healthy controls. Detailed baseline CTC results can be seen in table 3.

Correlation between CTCs and Clinicopathological Features

The presence of CTCs in the PB of pancreatic cancer patients at baseline did not correlate with gender, stage, tumor size, lymph nodes or metastasis. However, CTC positivity was slightly correlated with a higher histological tumor grading.

Correlation between CTCs and PFS in Pancreatic Cancer Patients

The correlation between PFS and baseline CTC status in pancreatic cancer patients was compared with the Kaplan-Meier method, and differences were tested using the log-rank test. PFS was calculated for groups defined by the presence or absence of CTCs before initiating chemotherapy (fig. 2). Overall median PFS was 120.0 days (95% CI 89.4–150.6), while shorter median PFS was observed for patients who had at least one detectable tumor-associated transcript, compared with patients who were CTC negative. Median PFS time was 66.0 days (95% CI 44.8–87.2) for CTC-positive patients and 138.0 days (95% CI 124.1–151.9) for CTC-negative patients (p = 0.01, log-rank test).

Table 2. Clinicopathological characteristics of the pancreatic cancer patients

Variable		All pancreatic cancer patients	CTCs		p value
			positive	negative	
Total patients, n		34	16	18	
Age at study entry, years	median	66.9			
	range	55–74			
Gender	female	14	6	8	0.738
	male	20	10	10	
Tumor stage	II	4	2	2	0.389
	III	2	0	2	
	IV	28	15	13	
Tumor size	T2	5	1	4	0.395
	T3	14	7	7	
	T4	15	8	7	
Lymph nodes	N1	4	1	3	0.791
	N2	30	15	15	
Histology grade	G2	24	8	16	0.042
	G3	9	7	2	
	G4	1	1	0	
Metastasis	yes	28	14	14	0.660
	no	6	2	4	

In order to evaluate whether different marker combinations would also correlate with PFS, we calculated PFS for patients with different combinations of positive CTC markers, as follows: when only *KRT19* was positive, p = 0.068; when only *MUC1* was positive, p = 0.273; when only *EPCAM* was positive, p = 0.174; when only *CEACAM5* was positive, p = 0.196; when only *BIRC5* was positive, p = 0.044; when *KRT19* or *MUC1* was positive, p = 0.014; when *KRT19* or *EPCAM* was positive, p = 0.042; when *MUC1* or *EPCAM* was positive, p = 0.051, and when *KRT19, MUC1* or *EPCAM* was positive, p = 0.006.

Discussion

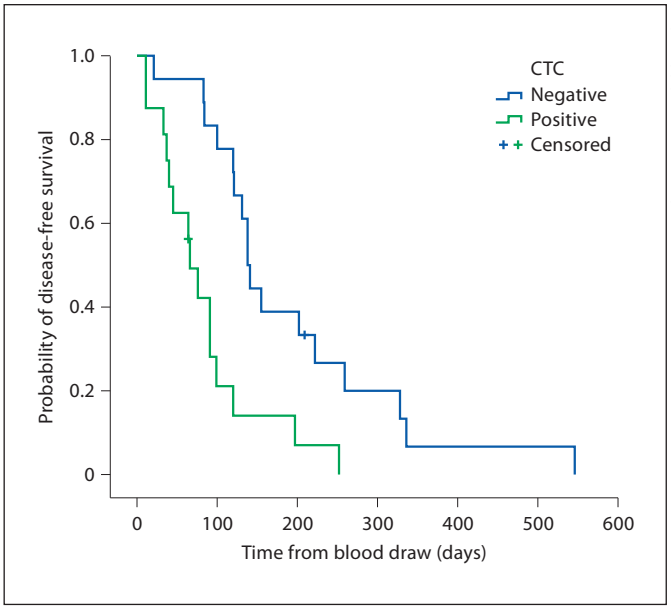
To the best of our knowledge, here we report the first study using mucin 1- and EpCAM-based immunomagnetic enrichment, followed by real-time RT-PCR analysis of *KRT19*, *MUC1*, *EPCAM*, *CEACAM5* and *BIRC5,* as a way to detect CTCs in PB of pancreatic cancer patients and to evaluate their prognostic effect.

Table 3. Detailed CTC data for the analyzed pancreatic cancer patients and healthy blood donor controls

Patient ID	Response	CTC status	CTC Cq value cutoff					
			ACTB (24.0–30.0)	*KRT19* (36.0)	*MUC1* (37.1)	*EPCAM* (36.0)	*CEACAM5* (37.8)	*BIRC5* (35.0)
66.1	PD	P	28.9	35.4	36.2	35.5	n.s.	n.s.
76.2	PD	N	27.3	n.s.	n.s.	n.s.	n.s.	n.s.
77.1	PD	N	26.3	n.s.	n.s.	n.s.	n.s.	n.s.
113.1	death	P	29.3	n.s.	n.s.	33.1	n.s.	35.1
136.2	PD	N	26.9	n.s.	39.1	n.s.	n.s.	35.8
148.1	PD	N	27.8	n.s.	39.1	n.s.	n.s.	n.s.
154.1	PD	N	29.2	n.s.	n.s.	n.s.	n.s.	35.7
154.7	PD	N	29.3	n.s.	n.s.	n.s.	n.s.	n.s.
167.1	PD	P	26.5	n.s.	36.3	34.1	36.9	34.9
179.1.1	PD	P	28.5	n.s.	n.s.	n.s.	n.s.	33.9
179.4	PD	N	27.4	n.s.	n.s.	n.s.	n.s.	n.s.
203.1	death	P	28.1	33.6	n.s.	34.9	n.s.	35.9
209.1	PD	N	27.6	n.s.	n.s.	n.s.	n.s.	n.s.
210.1	PD	P	27.3	31.9	n.s.	n.s.	n.s.	34.5
211.1	PD	N	28.4	n.s.	39.3	n.s.	n.s.	35.9
231.1	death	P	27.5	35.8	n.s.	n.s.	n.s.	n.s.
242.1	death	N	29.2	n.s.	n.s.	n.s.	n.s.	n.s.
246.1	death	P	27.5	34.0	n.s.	n.s.	n.s.	35.6
261.1	death	N	27.2	n.s.	n.s.	36.7	n.s.	n.s.
262.1	PD	P	26.7	n.s.	34.7	33.3	n.s.	33.8
267.1	death	N	28.2	n.s.	n.s.	n.s.	n.s.	n.s.
275.1	PD	N	28.8	n.s.	n.s.	n.s.	n.s.	n.s.
278.1	death	N	27.6	n.s.	n.s.	n.s.	n.s.	n.s.
295.1	PD	P	25.6	34.7	35.0	34.2	n.s.	n.s.
297.1	PD	N	28.4	n.s.	n.s.	n.s.	n.s.	n.s.
298.1	PD	P	25.8	n.s.	35.0	34.0	n.s.	35.2
299.1	PD	N	28.9	n.s.	n.s.	n.s.	n.s.	n.s.
300.1	death	P	27.6	n.s.	n.s.	36.6	n.s.	34.8
313.1	SD	N	28.9	n.s.	n.s.	n.s.	n.s.	n.s.
330.1	PD	P	29.1	34.9	n.s.	n.s.	n.s.	n.s.
345.1	PD	P	29.0	n.s.	37.0	n.s.	n.s.	n.s.
356.1	PD	P	28.6	n.s.	35.7	36.9	n.s.	n.s.
369.1	SD	P	27.8	n.s.	n.s.	35.0	n.s.	34.1
211.8	PD	N	29.0	n.s.	n.s.	n.s.	n.s.	n.s.
Healthy blood donor controls[1]			28.6 ± 1.2	n.s.	38.5 ± 0.6	36.9 ± 0.8	n.s.	35.7 ± 0.5

In the healthy blood donor controls, 7 probes gave a positive signal for *MUC1*, 11 probes for *EPCAM* and 6 for *BIRC5*. The mean ± SD values were calculated using only these probes. All the Cq values obtained in the healthy blood donor controls were under the defined CTC positivity cutoffs. PD = Progressive disease; SD = stable disease; P = positive; N = negative; n.s. = no signal of the gene found during the 40 PCR cycles.

[1] Cq values represent the mean ± SD of the 40 analyzed samples.



Color version available online

Fig. 2. PFS of pancreatic cancer patients with and without CTCs in 10 ml of blood before therapy (CTC positivity is defined by positivity of at least one marker gene). In the present study, the time to event data was measured as the time between the baseline of the blood sampling for CTC analysis and the documentation of death or first tumor progression based on clinical and radiological studies. Patients who were alive and progression free at the time of analysis were censored using the time between the baseline CTC assessment and their most recent follow-up evaluations.

Despite the progress in pancreatic cancer treatment by the application of new chemoradioimmunotherapy protocols, the high recurrence rate is the most limiting factor for the improvement of patients' prognosis [16]. Current methods for the detection of recurrence or metastasis in the postoperative period rely on serum tumor markers [17, 18] and imaging modalities, which include radiography, computed tomography, magnetic resonance imaging and sonography [19, 20]. However, these prognostic methods lack sensitivity and often do not allow for a more personalized approach to cancer treatment. Therefore, more sensitive staging methods capable of identifying patients who are destined to progress quickly or would benefit from more aggressive therapy are urgently needed for pancreatic cancer. Minimal residual disease caused by the spread of tumor cells into the circulation is discussed as a major reason for early metastases and local recurrence in pancreatic cancer. In recent years, only a few studies employing different methodologies for CTC isolation and detection [12, 21–

27] have focused on the isolation and identification of CTCs in PB of pancreatic cancer patients, with some of them reporting contradictory results concerning the prognostic significance of CTCs. Soeth et al. [25] detected CK20-positive mRNA transcripts in the PB of 33.8% (52/154) of pancreatic cancer patients before operation and showed a statistically significant relationship between overall survival and the detection of CTCs in PB. Zhou et al. [26] reported that the combined analysis of C-MET, h-TERT, CK20 and CEA could detect CTCs in the PB blood of 100% (25/25) of pancreatic cancer patients before operation, radiotherapy and chemotherapy, with 100% specificity. Finally, Sergeant et al. [27] reported that no association was found between EpCAM positivity in PB of pancreatic ductal adenocarcinoma patients (n = 48) and cancer-specific or disease-free survival; nor were significant correlations found between clinicopathological variables and perioperative EpCAM positivity.

In the present pilot study, we report the development of an immunomagnetic/real-time RT-PCR assay for CTC detection and characterization. Our data suggest that CTCs identified by this methodology can serve as a prognostic factor for pancreatic cancer patients beginning systemic treatment. The assay described herein provides a specific and sensitive method for isolation and identification of CTCs in PB of pancreatic cancer patients. CTCs were immunomagnetically enriched by coupling magnetic beads to the specific antibodies VU1D9 and BM7, with high affinity for the antigens EpCAM and mucin-1, respectively. By adding a second antibody targeting mucin-1, we have increased the chances for CTC isolation in comparison with solely EpCAM-based enrichment systems. All EpCAM-based assays share the same limitation, namely that EpCAM is expressed in most but not all tumors [28]. Several studies have found that EpCAM can be either upregulated or downregulated during cancer progression and metastasis [29, 30]. Moreover, the real-time RT-PCR assay developed here showed high specificity, as none of the healthy controls were found to be positive for the multimarker gene panel. In addition, by preparing a standard curve for each gene, we determined the linearity, efficiency and analytical sensitivity of the assay. Subsequently, Cq cutoff values for each gene were created, so as to guarantee that no Cq value above the corresponding reliable analytical limit of detection of the assay was considered positive [15].

When applied in the clinic, the present assay revealed that 47.1% of pancreatic cancer patients showed amplification of at least one tumor-associated marker mRNA in their PB sample. Furthermore, our results demonstrate that the presence of at least one clearly detectable tumor-associated transcript in the PB of pancreatic cancer patients is a strong predictive factor for shorter PFS. Patients who were CTC positive before treatment had significantly reduced disease-free survival time compared with patients who were CTC negative. Moreover, CTC findings were also correlated with tumor grading. As expected, we observed that the CTC detection rate was significantly increased when a multimarker panel was applied compared with the results obtained using single markers. Metastatic tumors are particularly heterogeneous, and therefore, the combination of marker genes can compensate for variations in individual marker expression, increasing the chances of CTC detection [31]. Moreover, multimarker analysis of CTCs allows for characterization of these malignant cells in terms of aggressiveness and phenotype [32]. This may further promote the selection of the most effective treatment and the creation of truly tailored therapy regimes.

The limitations of this work must be considered. The study population was relatively small, which may influence the interpretation of the results. However, small well-designed studies are of great value as they can provide results quickly and can thus become part of a first selection in order to design further, larger confirmatory studies.

In conclusion, our results suggest that in addition to the current prognostic methods for pancreatic cancer, CTC analysis represents a potential complementary tool for prediction of patient outcome and may ultimately enable the creation of tailored therapy and improved patient care.

Disclosure Statement

All the authors declare that they have no conflict of interests.

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