PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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CLINICAL RESEARCH

Detection of disseminated pancreatic cells by amplification of cytokeratin-19 with quantitative RT-PCR in blood, bone marrow and peritoneal lavage of pancreatic carcinoma patients

Katrin Hoffmann, Christiane Kerner, Wolfgang Wilfert, Marc Mueller, Joachim Thiery, Johann Hauss, Helmut Witzigmann

Katrin Hoffmann, Christiane Kerner, Johann Hauss, Helmut Witzigmann, Department of Visceral, Transplantation, Thoracic and Vascular Surgery, University of Leipzig, Germany
Wolfgang Wilfert, Marc Mueller, Joachim Thiery, Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University of Leipzig, Germany
Correspondence to: Dr. Katrin Hoffmann, Department of General Surgery, Ruprecht Karls University of Heidelberg, Im Neuenheimer Feld 110, Heidelberg 69120, Germany. katrin.hoffmann@med.uni-heidelberg.de
Telephone: +49-6221-566110 Fax: +49-6221-564215
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Abstract

AIM: To evaluate the diagnostic potential of cytokeratin-19 (CK-19) mRNA for the detection of disseminated tumor cells in blood, bone marrow and peritoneal lavage in patients with ductal adenocarcinoma of the pancreas.

METHODS: Sixty-eight patients with pancreatic cancer (n = 37), chronic pancreatitis (n = 16), and non-pancreatic benign surgical diseases (n = 15, control group) were included in the study. Venous blood was taken preoperatively, intraoperatively and at postoperative d 1 and 10. Preoperative bone marrow aspirates and peritoneal lavage taken before mobilization of the tumor were analyzed. All samples were evaluated for disseminated tumor cells by CK-19-specific nested-PCR and quantitative fluorogenic RT-PCR.

RESULTS: CK-19 mRNA expression was increased in 24 (64%) blood samples and 11 (30%) of the peritoneal lavage samples in the patients with pancreatic cancer. In 15 (40%) of the patients with pancreatic cancer, disseminated tumor cells were detected in venous blood and bone marrow and/or peritoneal lavage. In the peritoneal lavage, the detection rates were correlated with the tumor size and the tumor differentiation. CK-19 levels were increased in pT3/T4 and moderately/poorly differentiated tumors (G2/G3). Pancreatic cancer patients with at least one CK-19 mRNA-positive sample showed a trend towards shorter survival. Pancreatic cancer patients showed significantly increased detection rates of disseminated tumor cells in blood and peritoneal lavage compared to the controls and the patients with chronic pancreatitis.

CONCLUSION: Disseminated tumor cells can be detected in patients with pancreatic ductal adenocarcinoma by CK-19 fluorogenic RT-PCR. In peritoneal lavage, detection rate is correlated with tumor stage and differentiation. In the clinical use, CK-19 is suitable for the distinction between malignant and benign pancreatic disease in combination with other tumor-specific markers.

Key words: Tumor cell dissemination; Pancreatic cancer; Cytokeratin-19

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INTRODUCTION

Pancreatic cancer is one of the top five causes of cancer death in the Western world. The 5-year survival rates are around 4%[1,2]. Tumor resection is associated with prolonged survival and postoperative adjuvant chemotherapy may further improve long-term results[3,4]. However, curative treatment of most patients fails due to local recurrence and hepatic metastases occurring within two years after surgery[5,6]. Occult micro-metastases caused by disseminated tumor cells are the most limiting factor for the improvement of mortality rates. Their influence on prognosis and development of new therapeutic strategies has not yet been completely elucidated.

Disseminated tumor cells are not ascertainable with current staging methods. Additional to cytology, immunohistochemical analysis is the standard for identification of tumor cells. Despite improvement of detection rates by conventional cytology, conflicting result regarding the prognostic relevance have been reported[7]. Several studies focused on molecular biological approaches for the qualitative or semi-quantitative verification of tumor cell dissemination in pancreatic cancer[8-12]. Reverse transcriptase polymerase chain reaction (RT-PCR) has a high sensitivity and allows the identification of approximately 1 tumor cell in 10^7 normal peripheral mononuclear blood cells[13]. Using qualitative PCR methods, varying frequency of gene transcripts and false positive results have been reported[14-16]. Therefore, tumor cell detection in patients with ductal adenocarcinoma of the pancreas is still a matter of debate.

Nowadays CEA and CA 19-9 are established as clinical markers for pancreatic cancer. However, expressions of these antigens have also been reported in cholangitis, chronic pancreatitis and various gastrointestinal tumors[17]. Various studies used cytokeratin-19 (CK-19) for pancreatic cell detection. CK-19 has been identified as a reliable marker for epithelial cell differentiation[18]. It is specific for undifferentiated pancreatic ductal cells and homogenously expressed at high levels in primary pancreatic adenocarcinoma and pancreatic carcinoma metastases[19,20]. CK-19 is not expressed in hematopoetic cells and therefore suited for detection of disseminated pancreatic cells in the peripheral blood[19,21].

Here, we report on the expression of CK-19 mRNA in blood, bone marrow and peritoneal lavage in 68 patients. Nested-PCR and quantitative fluorogenic RT-PCR were used to detect dissemination of pancreatic cells in patients with pancreatic cancer, chronic pancreatitis and non-pancreatic benign surgical diseases. The purpose of this study was to evaluate the potential of qualitative nested-PCR and quantitative RT-PCR for the detection of disseminated pancreatic cells and the differentiation between chronic pancreatitis and pancreatic cancer.

MATERIALS AND METHODS

Patients

This prospective study was approved by the Ethical Commission of the University of Leipzig and informed written consent was obtained from each patient. Peripheral blood samples, bone marrow aspirations and peritoneal lavage of 68 patients were analyzed. Thirty-seven patients with histologically confirmed primary pancreatic cancer, 16 patients with chronic pancreatitis and 15 controls with non-pancreatic benign surgical diseases participated in the study. In all patients, diagnosis was confirmed by the resected specimen or tumor biopsy for non-resectable cancers (n = 11). Patients with pancreatic cancer were staged according to the UICC guidelines 2005[22]. According to the UICC stage, 4 (10%) patients had stage Ⅰ, 3 (8%) stage Ⅱ, 19 (51%) stage Ⅲ, and 11 (29%) stage Ⅳ pancreatic cancer. The survival time of the patients ranged between 3 and 48 mo (median survival: 12 mo). Patients with cancer of the common bile duct, the ampulla of Vater or the duodenum were excluded from the study.

Samples

Four blood samples were obtained from each patient through central venous catheter 30 min preoperatively, intraoperatively after mobilization of the pancreas and 24 h as well as 10 d after the operation. Bone marrow samples were obtained after induction of general anesthesia by aspiration from the iliac crest and heparinized. Peritoneal lavage was performed immediately after exploration of the abdominal cavity. About 500 mL of sterile isotonic sodium chloride solution was instilled, removed after irrigation and then EDTA buffer was added.

RNA extraction and cDNA synthesis

All samples were diluted and washed with 10 mL of erythrocyte lysis buffer. The mononuclear cell fraction was isolated by Ficoll-Isopaque (Amersham, Braunschweig, Germany). Total RNA was extracted using guanidinium-isothiocyanate-phenol-chloroform-based method (Trizol, Gibco BRL, Life Technologies, Gaithersburg, USA). RNA integrity was checked electrophoretically and quantified spectrophotometrically. First strand cDNA was generated from 3 μg of total RNA diluted with 10 μL of RNase-free water using 1 μL of Random Hexamer Primer (Roche Diagnostics, Basel, Switzerland) and incubated for 10 min at 68℃. After chilling on ice, 7 μL of master mixture, according to Superscript Ⅱ kit (Invitrogen, Carlsbad, USA), was added and after 60 min of incubation at 42℃, the reaction was inactivated for 10 min at 80℃.

Qualitative nested-PCR

Qualitative analysis of CK-19 expression was carried out using nested-PCR. Two different pairs of primer (n-PCR1/n-PCR2) were designed. The second set of primers amplifies inside the amplification sites of n-PCR1 primers to improve the specificity of PCR. Then 5 μL of cDNA was diluted with 45 μL of mixture containing 1 μL of each n-PCR1 primer, 10 × PCR buffer, dNTP PCR, AmpliTaq polymerase (Perkin Elmer Life and Analytical Sciences, Boston, USA) and DNase-free water for first round PCR. The conditions for PCR step one and two were one cycle at 95℃ for 3 min, followed by 40 cycles at 57℃ for 20 s, 72℃ for 50 s and a final extension at 72℃ for 10 min. For second round PCR, 1 μL of aliquot of the first round PCR product was added to 49 μL of master mixture containing 1 μL of each n-PCR2 primer. Two negative controls were included per run. Ten microliters of all PCR products were electrophoresed on 20 g/L agarose gels and visualized after ethidium bromide staining (Table 1).

Quantitative RT-PCR

CK-19 and housekeeping genes beta-actin primers and fluorogenic probes were designed using Primer Express software (Primerexpress Version 2.0, Perkin Elmer) (Table 1). PCR primers and probes have been positioned to span exon-intron boundaries. Amplification and detection of CK-19 a and b pseudogen are unlikely because probe and

Table 1 Sequences for quantitative RT-PCR and nested-PCR primers and flourescent probes

	Forward primer	Reverse primer	Length (bp)
TaqMan primer CK-19	5'-GAAGGCCTGAAGGAAGAGCTG-3'	5'-CCTCCCACTTGGCCCCT-3'	80
TaqMan primer beta-actin	5'-TCCAGAGGCGCTCTTCCA-3'	5'-CGCACTTCATGATCGAGTTGA-3'	86
Vector primer CK-19	5'-AACTCCAGGATTGTCCTGCAG-3'	5'-TCCCGGTTCAATTCTTCAGTC-3'	401
Vector primer beta-actin	5'-GCACCACTGGCATTGTCATG-3'	5'-CCACACGGAGTACTTGCGC-3'	581
Nested-PCR primer CK-19	5'-AACTCCAGGATTGTCCTGCAG-3'	5'-TCCCGGTTCAATTCTTCAGTC-3'	401
Nested-PCR primer CK-19	5'-GAAGGCCTGAAGGAAGAGCTG-3'	5'-CCTCCCACTTGGCCCCT-3'	80
Probe CK-19	5'-CCTACCTGAAGAAGAACCATGAGGAGGAAATCAGTA-3'		
Probe beta-actin	5′-CCTCCTTCCTGGGCATGGAGTCCTG-3′		

primer contain several mismatches. mRNA quantification was carried out using the one tube, one enzyme fluorogenic RT-PCR protocol[23]. Ten microliters of cDNA was diluted with 40 μL of master mixture containing TaqPolymerase, dNTP mixture and 10 × AmpliTaq buffer A, 200 nmol/L probe, 900 nmol/L primer and 25 mmol/L $MgCl_2$. After 10 min of denaturation at 95℃, PCR was carried out for 40 cycles at 95℃ for 15 s and extension at 60℃ for 60 s in the presence of the probe. RT-PCR monitoring was achieved by measuring the fluorescent signal of the probe at the end of the annealing phase of each cycle. mRNA quantification was recorded and analyzed with the ABI 7700 Prism Sequence detection system (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Two no-template controls were used to monitor contamination in every run. Accurate quantification was achieved through generation of standard curves by serial dilution of CK-19 and beta-actin RNA transcribed by RNA polymerase. The sensitivity of the technique was evaluated by serial control analysis obtained in dilution experiments with CK-19-expressing cancer cell lines (CaPan2, PANC1). It was possible to detect the CK-19 mRNA expression of one cell of the cell lines in 1 mL of normal peripheral blood.

Evaluation criteria

For nested-PCR, the samples were tested twice. If a CK-19 signal was detected, the sample was judged positive. The fluorogenic RT-PCR assay was done twice for each sample. The average value of both duplicates for each sample was used as quantitative value. Samples were excluded from the investigation if the expression of the house-keeping gene beta-actin was below 10^6 copies. The ratio of copies of CK 19 mRNA per 10^6 copies of beta-actin mRNA was used for further analysis. The introduction of a cut-off was required, due to high illegitimate background transcription in the control group. The samples which exceeded the maximum value of the CK-19 mRNA expression in the control group were defined as CK-19 mRNA-positive.

Statistical analysis

Mann-Whitney rank sum test was used for quantitative analysis to asses the differences in the means. Fisher's exact test was used for qualitative analysis. Two-tailed *P* value less than 0.05 was considered statistically significant. Kaplan-Meier and log-rank test were used for analysis of survival. Statistical analysis was carried out using SigmaStat 1.0 software (Jandel Scientific Corp., Erkrath, Germany).

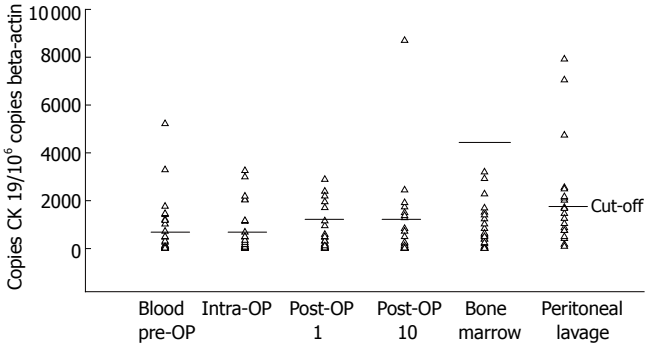

Figure 1 Quantitative values of cytokeratin-19 transcription in blood, bone marrow and peritoneal lavage of the patients with pancreatic cancer. [1]Cut-off: Maximal expression of CK-19 mRNA in healthy control patients.

RESULTS

Qualitative nested-PCR

CK-19 mRNA expression was detectable in the blood in 65% (24/37) of the patients with pancreatic cancer, 53% (8/16) of the patients with chronic pancreatitis and 18% (3/15) of the control group. The analysis of the bone marrow samples revealed detectable CK-19 mRNA in 56% (21/37) of pancreatic cancer patients, 46% (7/16) of chronic pancreatitis patients and 66% (10/15) of the control group. In 51% (19/37) of the patients with pancreatic cancer and 46% (7/16) of the patients with chronic pancreatitis and 46% (7/15) of the control group, CK-19 mRNA was detectable in the peritoneal lavage. There was no statistically significant difference between the groups.

Quantitative real-time RT-PCR

Blood, bone marrow and peritoneal lavage samples of 37 patients with pancreatic cancer were analyzed by CK-19 fluorogenic RT-PCR. CK-19 mRNA transcripts were detected in 70% (26/37) of blood samples, 67% (25/37) of bone marrow aspirates and 54% (20/37) of peritoneal lavage samples. In 40% (15/37) of the patients, PCR result was obtained from at least two compartments. We found that 64% (24/37) of the blood samples and 30% (11/37) of the peritoneal lavage samples showed CK-19 mRNA expression above the cut-off value and were defined as CK-19 mRNA-positive (Figure 1).

The CK-19 signal exceeded the cut-off value in 21% (8/37) of the pre-operatively and 19% (7/37) of the

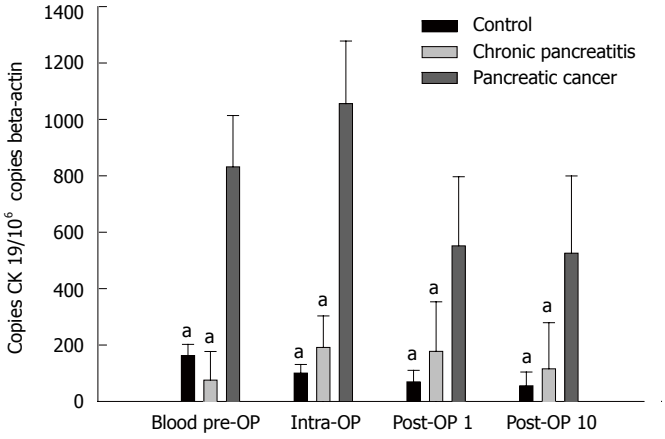

Figure 2 Quantitative analysis of CK-19 expression in blood (mean ± SD). $^{a}P < 0.05$ *vs* pancreatic cancer.

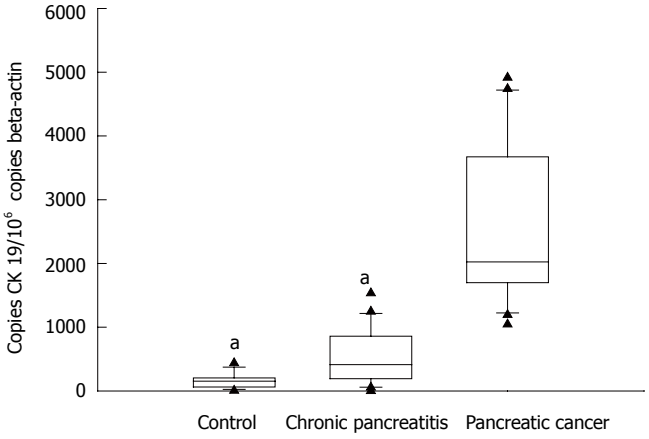

Figure 3 Quantitative CK-19 expression in peritoneal lavage of the patients with pancreatic cancer (mean ± SD, $^{a}P < 0.05$ *vs* pancreatic cancer).

intraoperatively taken blood samples. Likewise, 13% (5/37) of the samples taken at the first postoperative day and 10% (4/37) of the samples taken at the 10th postoperative day showed expression of CK-19 mRNA. The median mRNA expression was significantly increased in the patients compared to the control group at the four time points ($P < 0.05$) (Figure 2). The CK-19 levels showed a trend to increase intraoperatively and decrease at the 1st and 10th postoperative days below the pre-operative level ($P = 0.07$). We found 64% (24/37) of the bone marrow samples exhibited detectable CK-19 mRNA expression. None of the samples exceeded the CK-19 mRNA expression of the control group. The detection rate was not statistically different. In the peritoneal lavage, CK-19 signal was detected in 54% (20/37); 30% (11/37) of these samples exceeded the maximum level of the control group and were found to be CK-19 mRNA-positive. Compared to the control group, the median CK-19 mRNA expression was at least 10-fold increased (533 ± 121 copies CK-19/10^6 copies beta-actin *vs* 6262 ± 557 copies CK 19/10^6 copies beta-actin, $P < 0.01$) (Figure 3) .

The correlation analysis of marker detection and stage of disease was performed for blood, bone marrow and peritoneal lavage. In peritoneal lavage, CK-19 mRNA levels correlated with the tumor size and were increased 3 times in the patients with a pT3/pT4 tumor compared to the patients with pT1/pT2 tumors (874 ± 87 copies/10^6 copies beta-actin *vs* 2884 ± 473 copies/10^6 copies beta-actin ($P < 0.05$). Detection rates were increased for the patients with moderate or poorly differentiated tumors (pG2/pG3) compared to the patients with well differentiated tumors (pG1) (3560 ± 302 copies CK-19/10^6 copies beta-actin *vs* 1055 ± 88 copies CK-19/10^6 copies beta-actin, $P < 0.05$) (Figure 4). The detection rates were not significantly different regarding the N stage or in blood and bone marrow samples. At the endpoint of the study, 14 patients had died of metastasized disease or tumor recurrence; 57% (8/14) of these patients had at least one CK-19 positive sample. No significant difference in the median survival was observed between the patients with positive CK-19 mRNA expression and negative CK-19 mRNA expression (10 mo *vs* 15 mo, $P = 0.15$).

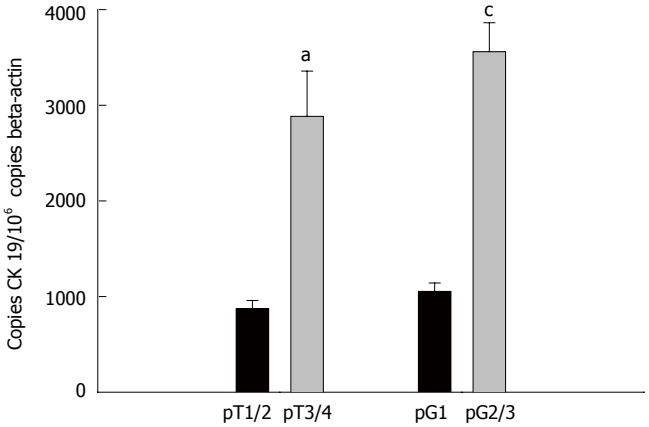

Figure 4 Quantitative CK-19 expression in peritoneal lavage of the patients with tumors (mean ± SD). $^{a}P < 0.05$ *vs* pT1/2; $^{c}P < 0.05$ *vs* pG1.

Blood, bone marrow and peritoneal lavage samples of 31 patients without malignancies (15 patients of the control group and 16 patients with chronic pancreatitis) were evaluated. CK-19 expression was detectable in 80% (12/15) of the samples taken from the control group. The highest detectable CK-19 signal was used as the base line for the cut-off values which were as follows: blood preoperatively 1064 copies CK-19 per 10^6 copies beta-actin, intraoperatively 984 copies CK-19 per 10^6 copies beta-actin, 1st and 10th postoperative days 1179 copies CK-19 per 10^6 copies beta-actin; bone marrow 4200 copies CK-19 per 10^6 copies beta-actin; and peritoneal lavage 1600 copies CK-19 per 10^6 copies beta-actin.

CK-19 mRNA could be detected in 80% (12/16) of the blood samples, 53% (8/16) of the bone marrow aspirates and 60% (9/16) of peritoneal lavage of the patients with chronic pancreatitis. No analyzed sample exceeded the maximal CK-19 mRNA expression of the control group. We observed a five-fold lower CK-19 signal in the peripheral blood compared to the patients with pancreatic cancer (142 ± 138 copies/10^6 copies beta-actin *vs* 741 ± 278 copies/10^6 copies beta-actin). The gene expression was significantly different at the four investigated time points ($P < 0.01$) (Figure 2). Analysis

of the bone marrow samples did not reveal any obvious difference in the CK-19 mRNA expression between the investigated groups. Compared to the patients with pancreatic cancer, the median CK-19 mRNA expression in the peritoneal lavage was 10-fold lower in the patients with chronic pancreatitis (261 ± 134 copies/10^6 copies beta-actin *vs* 3319 ± 484 copies/10^6 copies beta-actin, $P < 0.01$).

DISCUSSION

The long-term survival rates for patients with pancreatic cancer remain low. Without treatment, the 5-year survival rates are 0.4% to 4%[1,24]. Up to 80% of patients develop recurrent disease within 2 years after tumor resection[5]. Despite progress in adjuvant therapy and application of new chemo-radio-immunotherapy protocols, the high recurrence rate is the most limiting factor for the improvement of the patients prognosis[3,25]. Therefore, more sensitive staging methods are needed for this kind of tumor. Minimal residual disease caused by the spread of tumor cells into the circulation either before or during surgery is discussed as a main reason for early metastases and local recurrence in pancreatic cancer[26,27]. To detect tumor cells in the circulation, several studies have focused on cytokeratins and their potential role as tumor-specific markers[11,12,20,28-32]. However, conventional cytology, immunohistochemistry and molecular biological approaches reported conflicting results regarding the prevalence of CK-19 positive disseminated tumor cells and the impact on the prognosis of patients[12,33]. A reason for this is the target gene expression by non-neoplastic cells. False-positive results caused by illegitimate background transcription of CK-19 as seen in this study pose a major problem for qualitative PCR analysis. Gene transcription of CK-19 in healthy control groups and patients with benign disease has been reported in up to 80% of the analyzed samples[14,16]. In order to prevent false-positive results and increase the specificity, samples in this study were analyzed by quantitative RT-PCR. This approach facilitated the definition of cut-off values representing the maximal target gene expression in the control group. Regarding the results of nested-PCR in our study group, the qualitative PCR analysis of CK-19 expression can not be recommended for the detection of disseminated pancreatic cells.

Despite detection rates between 4%-100% using K-ras, CEA and cytokeratines as markers, up to now, tumor cell dissemination in the blood is not identified as an independent prognostic marker in pancreatic cancer[12,34-39]. In our sample series, 64% of peripheral blood samples taken from the patients with pancreatic cancer were detected to be CK-19-positive by quantitative fluorogenic RT-PCR. To reflect the dynamics of tumor cell dissemination during surgical intervention, blood samples were analyzed at four different time points showing target gene detection rates significantly higher compared to the control group. As described for K-ras and CEA mRNA, the amount of detectable CK-19 transcripts showed a trend to increase intraoperatively and decrease postoperatively below the preoperative level[26,36,37]. In consistent with Aihara *et al*[29], the CK-19 mRNA expression in the peripheral blood in our study showed higher levels.

Various studies have focused on the detection of disseminated pancreatic tumor cells in the bone marrow. Though positive detection rates have been reported between 24% and 58% for different markers, the tumor cell detection in the bone marrow does not correlate with tumor stage and is not an independent prognostic marker[11,12,34,35]. There is only one study by Soeth *et al*[36] demonstrating a benefit in survival in patients with CK-20-negative bone marrow aspirates. The data obtained in our study confirm the results of Dimmler *et al*[14], who dissuade from use of CK-19 for analysis of bone marrow due to high levels of illegitimate background expression in healthy controls. In contrast, peritoneal lavage was identified to be suitable to represent the actual state of tumor dissemination. The immunohistochemical analysis of the peritoneal lavage reported detection rates between 20% and 58% for marker-positive pancreatic cells[34,35,37-39]. Compared to the analysis by Inoue *et al*[40] who reported 10% of K-ras mRNA-positive cells, our detection rate of CK-19 positive cells was 30%. To the best of our knowledge, this sample series is the first report that shows a correlation between the CK-19 mRNA expression in the peritoneal lavage and the tumor size and tumor differentiation. A correlation between positive tumor cell detection and impaired patient survival as reported for cytological analysis by Yachida *et al*[37] and Makary *et al*[41] could not be detected.

Besides the detection of dissemination of neoplastic cells, the differentiation between malignant and benign pancreatic disease is of major interest for clinicians. Up to now, several markers such as K-ras, p53 and CEA failed as serum marker for a differential diagnosis[17,42]. CK 19-9 is the best characterized serum marker but has no absolute specificity. This is the first study that evaluates CK-19 mRNA expression in different compartments for the potential to differentiate between pancreatic cancer and chronic pancreatitis. In our study, the patients with pancreatic cancer showed an unequivocally increased CK-19 expression compared to the patients with chronic pancreatitis. Ten-fold higher expression levels in the peripheral blood as well as in the peritoneal lavage from the pancreatic cancer patients compared to the chronic pancreatitis patients indicated that quantitative fluorogenic RT-PCR is suited for the distinction between malignant and benign pancreatic disease.

In conclusion, our data suggest that CK-19-positive tumor cells can be detected in patients with pancreatic carcinoma in venous blood and peritoneal lavage by using fluorogenic RT-PCR. The prevalence of isolated tumor cells in peritoneal lavage increases significantly with the tumor stage and differentiation. The application of highly sensitive RT-PCR technique may improve the staging of patients and the monitoring of the residual tumor cell burden within the context of adjuvant systemic therapies. For the clinical use, the combination of tumor-specific markers is indispensable to increase the specificity of this test.

COMMENTS

Background

Pancreatic cancer is one of the top five causes of cancer death in the Western world. Occult micro-metastases caused by disseminated tumor cells are the most limiting factor for the improvement of mortality rates.

Research frontiers

Additional to cytology, immunohistochemical analysis is the standard for identification of disseminated tumor cells. Nevertheless, conflicting results regarding the prognostic relevance have been reported. Molecular biological approaches, such as reverse transcriptase polymerase chain reaction (RT-PCR), have a high sensitivity and specificity and might be more suitable for the analysis of disseminated tumor cells.

Innovations and breakthroughs

Various studies used cytokeratin-19 (CK-19) for pancreatic cell detection that has been identified as a reliable marker for epithelial cell differentiation and is homogenously expressed at high levels in primary pancreatic adenocarcinoma and pancreatic carcinoma metastases. Until now, CK-19 mRNA expression has not been identified as an independent prognostic indicator in pancreatic cancer.

Applications

Our data suggest that CK-19-positive tumor cells can be detected in patients with pancreatic carcinoma in venous blood and peritoneal lavage by using fluorogenic RT-PCR. The prevalence of isolated tumor cells in peritoneal lavage increases significantly with the tumor stage and differentiation. The application of highly sensitive RT-PCR technique may improve the staging of patients and the monitoring of the residual tumor cell burden within the context of adjuvant systemic therapies. For the clinical use, the combination of tumor-specific markers is indispensable to increase the specificity of this test.

Peer review

The authors demonstrate that quantification of CK-19 seems to be a reliable marker for the differential diagnosis of ductal pancreatic cancer and for staging. The Material and Methods, Results and Discussion are adequate and the paper provides new information for the investigators in this field.

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