

# Occult tumour cells in peritoneal lavage are a negative prognostic factor in pancreatic cancer

Roman Havlik<sup>a,§</sup>, Josef Srovnal<sup>b,§</sup>, Dusan Klos<sup>a</sup>, Andrea Benedikova<sup>b</sup>, Martin Lovecek<sup>a</sup>, Mohamed Ghothim<sup>a</sup>, Dana Cahova<sup>b</sup>, Cestmir Neoral<sup>a</sup>, Marian Hajduch<sup>b</sup>

**Aims.** The aim of this study was to test the hypothesis that occult tumour cells in peritoneal lavage are a negative prognostic factor in pancreatic adenocarcinoma.

**Methods.** Real-time RT-PCR analysis of CEA, EGFR and hTERT transcript levels was used to identify occult tumour cells in peritoneal lavage samples from 96 pancreatic cancer patients.

**Results.** We found significant association between CEA expression levels in peritoneal lavage and clinical stage. We also found that EGFR transcript levels were higher in peritoneal lavage samples from patients with high grade tumours than in samples from patients with low grade tumours. Detection of CEA and/or EGFR occult tumour cell markers in the peritoneal lavage was associated with significantly shorter overall survival and increased hazard ratio for disease recurrence.

**Conclusions.** The results show that the presence of occult tumour cells in peritoneal lavage is a negative prognostic factor for survival in pancreatic cancer patients, and that detection of occult tumour cells using PCR-based methods can identify patients with advanced disease for whom radical surgery is likely to have little benefit.

**Key words:** pancreatic adenocarcinoma, occult tumour cells, peritoneal lavage, prognostic factor, RT-PCR

Received: February 2, 2012; Accepted: June 7, 2012; Available online: October 31, 2012  
<http://dx.doi.org/10.5507/bp.2012.061>

<sup>a</sup>Department of Surgery I, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic

<sup>b</sup>Laboratory of Experimental Medicine, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University Olomouc

<sup>§</sup>Both authors contributed equally to the work

Corresponding author: Marian Hajduch, email: [marian.hajduch@fnol.cz](mailto:marian.hajduch@fnol.cz)

## INTRODUCTION

As the most aggressive of all major cancers, pancreatic cancer is a significant medical problem facing current health-care systems. It has a very poor prognosis, the incidence is increasing and, despite advances in research, the mortality rate is not decreasing<sup>1</sup>. It is a disease that is often diagnosed at an advanced stage, when treatment options are limited. Distant metastases and peritoneal carcinomatosis are caused by early dissemination of tumour cells, either by haematogenic or lymphogenic pathways. The most common cause of local recurrence is perineural spread. No reliable screening programme is available yet to detect pancreatic cancer at an early stage. In addition, systemic therapy has limited efficacy and in most cases does not lead to a radical extension of patients' survival times. Some authors therefore consider the disease to be systemic from the start and a number of surgeons have rejected or rarely performed radical operations on the pancreas with therapeutic intent<sup>2</sup>. This view is surely overly pessimistic, especially for patients with curable resectable tumours. However, it does suggest that pancreatic cancer is highly invasive from the start, due to its localization and aggression.

Treatment of pancreatic cancer is multimodal. The only potentially curative approach is surgical R0-resection.

However, radical surgery is possible only for a minority of patients with this type of tumour. Five-year survival rates after radical resections for pancreatic cancer are still very low, in the range of 2-4% (ref.<sup>1,4</sup>). Reported significant negative prognostic factors for survival after resection include positive tumours in lymph nodes, high tumour grading, tumour sizes greater than 2.5 cm, the presence of tumours in the resection line, vascular invasion and tumour invasion through the capsule, which implies early local recurrence<sup>3,4</sup>.

Occult tumour cells are tumour cells in cancer patients that are invisible using conventional diagnostic methods. However, they can be detected using current molecular methods, notably real-time RT-PCR, with high sensitivity. The presence of occult tumour cells still has no clear clinical significance, but their presence in peritoneal lavage is indicative of advanced disease and poor prognosis<sup>5</sup>.

The surgical techniques and methodology for radical resection (the only potentially curative method of treating pancreatic cancer, as mentioned above) are fully standardized. No innovation, in terms of mini-invasive access or extent of radicality and lymphadenectomy, has led to increases in patient survival rates, despite intensive efforts. The options in terms of surgical therapy appear to have been exhausted. Thus, other treatment modalities must be sought at the molecular level. Notably, new knowledge of the etiopathogen-

esis and mechanisms of pancreatic carcinogenesis may lead to the discovery of new chemotherapeutic, biological, or even gene therapy treatments. From this perspective, assessment of the potential utility of occult tumour cells as a diagnostic and prognostic factor is highly important. The aim of this study was to test the hypothesis that occult tumour cells in peritoneal lavage are a negative prognostic factor in pancreatic adenocarcinoma.

## PATIENTS AND METHODS

### Pancreatic cancer patients

The study included 96 patients (38 females, 58 males, between 37 and 84 years old, mean age 62.8 years, at the time of surgery) operated on at the 1<sup>st</sup> Department of Surgery at the University Hospital in Olomouc during the period 2007-2010 for pancreatic cancer at all clinical stages as defined by the Union for International Cancer Control (Table 1). The extent of the surgical procedures ranged from simple explorations with biopsy through bypass operations - choledochoduodenostomy and gastroenteroanastomosis - to radical surgical operations in the form of right-sided hemipancreatoduodenectomy for pancreatic head tumours. The standard procedure at the institution has become pylorus-preserving hemipancreatoduodenectomy, introduced in 1944 by Watson and modified by Longmire and Traverse in 1978. It is based on specimen removal and then the remnant is reanastomosed to the first jejunal loop, by end-to-end or end-to-side pancreatojejunostomy, then end-to-side hepaticojejunostomy and finally antecolic end-to-side duodenojejunostomy. According to final histopathological classifications, the surgical procedures are described as R0 resection (negative lymph nodes and negative resection margins), R1 resection (positive lymph nodes, but all positive lymph nodes were removed, negative resection margins), R2 resection (tumour residua in resection margins or remaining lymph nodes) and NR non-resections (advanced disease, not indicated for resection - exploration only or different non-resectional procedure). The study was approved by the Ethics Committee of the University Hospital and all individuals signed informed consent.

### Peritoneal lavage sampling

Peritoneal lavage samples were obtained from all 96 patients, using 100 mL of phosphate buffered saline (pH=7.2) during the surgery starting prior to resection or mobilization. The peritoneal lavage was aspirated into sterile bottles containing 1.5 mL of 0.5 M EDTA buffer and 10 mL of fetal bovine serum then immediately transported to the laboratory. After centrifugation at 4500 rpm for 4 min, the whole cellular pellet was used for RNA purification.

### RNA purification and reverse transcription

Total RNA from the peritoneal lavage samples was purified using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. For reverse transcription, 3 µg of total RNA was pre-incubated with Random Primers (Promega, Madison, WI, USA) and then transcribed using RevertAid Moloney Murine Leukemia Virus reverse transcriptase (Fermentas, Vilnius, Lithuania).

### Real-time RT-PCR

To amplify cDNA of carcinoembryonic antigen (CEA, NM 004363), epidermal growth factor receptor 1 (EGFR1, NM\_005228) and human telomerase reverse transcriptase (hTERT, NM\_198253), the following primers and probes were used: CEA-fw 5'-TAAGTGTGAC-CACAGCGACCC-3', CEA-rev 5'-GTTCCCATCAATCAGCCAAGAA-3' and CEA-probe 5'-ATGTCCTCTATGGCCAGACGACC-3' BHQ1-HEX (179 bp); EGFR-fw 5'-ACTTCAAACTGCACCTCCAT-3', EGFR-rev 5'-AATCAGCAAAACCCGTGTGATT-3' and EGFR-probe 5'-ACATCCTGCCGGTGGCATTAGG-3' BHQ1-HEX (149 bp); hTERT-fw 5'-ATGTCACGGAGACCACGTTT-3', hTERT-rev 5'-GCACCTCTTCAAGTGCTG-3' and hTERT-probe 5'-TGGAG-CAAGTTGCAAGCATTGGA-3' BHQ1-HEX (105 bp) (all from Generi-Biotech, Hradec Kralove, Czech Republic). Real-time RT-PCR amplification was performed using HotStart Taq Polymerase (AB Gene, Epsom, UK) and a Rotor Gene 3000 cyclor (Corbett Research, Sydney, Australia). Standards for absolute gene expression quantification were made by cloning specific PCR amplicons using the Topo TA Cloning System into the pCR 2.1-Topo plasmid (Invitrogen, Carlsbad, California, USA) according to the manufacturer's recommended protocol. The cut-off values

**Table 1.** Clinical and laboratory characteristics of the 96 pancreatic cancer patients enrolled in the study.

		Sex	Mean age		Median OS		Radicality			Grading			
		n	Female/ Male	at diagnosis (years) (min-max)		(months) (95% CI)		R0	R1	R2	1	2	3
Clinical Stage	1	7	0/7	57.7	(44-71)	23.2	(19.3; ND)	7	0	0	3	4	0
	2	21	9/12	62.4	(44-84)	21.6	(14.9; ND)	21	0	0	0	17	4
	3	34	10/24	64.2	(46-82)	11.9	(11.5; ND)	32	2	0	1	24	9
	4	74	34/40	62.5	(37-77)	6.8	(5.9; 9.5)	6	2	66	1	23	44
Total		136	53/83	63.0	(37-84)	10.3	(8.9; 11.5)	66	4	66	5	68	57

Note: grading was not assessed in 4 patients, where histopathological diagnosis was confirmed by fine needle aspiration biopsy only.

for occult tumor cell positivity in peritoneal lavage were selected by the optimization process so that positive patients differ in the majority of cases from their negative counterparts using Kaplan-Meier curves. The cut-off values are set at 500 copies of hTERT mRNA/ $\mu$ g RNA, 1000 copies of CEA mRNA/ $\mu$ g RNA and 1 000 000 copies of EGFR mRNA/ $\mu$ g RNA.

### Statistical analyses

Occult cell positivity in the lavage samples (evaluated as an expression of three marker epithelial genes) was correlated with clinicopathological characteristics (tumor size, clinical stage, affected lymph nodes, presence of distant metastases and extent of surgical procedure) and patient survival duration. The significance of the correlations was then assessed, setting  $\alpha=0.05$  as the threshold level for significance, using Statistica 10 (StatSoft, Tulsa, USA). The marker transcript levels were not normally distributed, according to a Shapiro-Wilk test, thus nonparametric (Kruskal-Wallis Anova and Mann-Whitney) tests were applied in subsequent analyses. Dependencies between the marker levels and other continuous variables were validated by testing the null hypothesis (lack of association) using Spearman's correlation analyses. To assess possible associations between patients overall survival (from the date of the operation and sample collection for each patient) and the markers, Kaplan-Meier methods and Gehan-Wilcoxon tests were used.

## RESULTS

Overall, 73 of 93 (67.9%) of the pancreatic cancer patients died and the overall median survival time was 10.3 months (three patients were lost during follow-up). In total, 40 of the 96 (41.7%) patients (in clinical stage I-III) underwent potentially curative surgery with R0 resection. At our centre, the 30-day postoperative mortality following radical resection is 2.5% (ref.<sup>4</sup>). The prognostic signifi-

cance of the presence of occult tumour cells in peritoneal lavage at the time of surgery was evaluated using RT-PCR analysis of CEA, EGFR and hTERT transcript levels.

### Occult tumour cells and patient characteristics

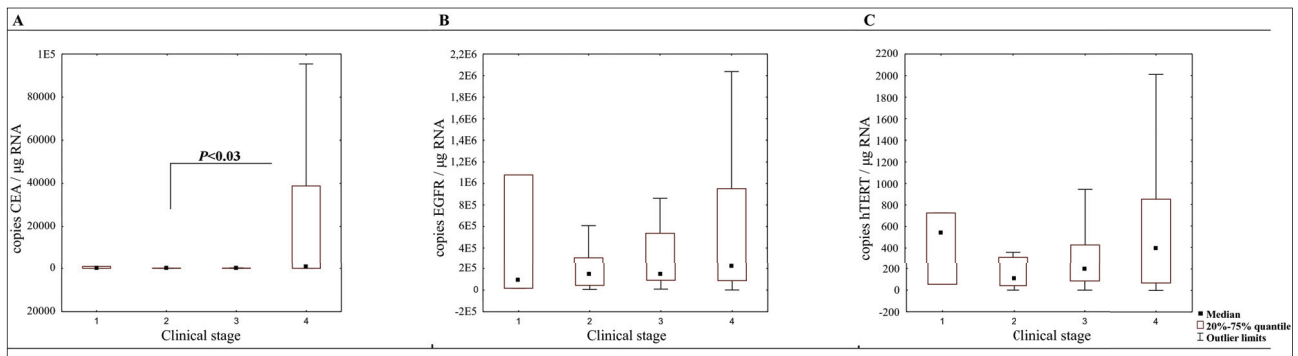
We detected associations in occult tumour cell positivity in peritoneal lavage, based on levels of the CEA, EGFR and hTERT markers, with clinical stage, tumour grading and surgical radicality. Patients with advanced disease, higher tumour grades, R2 resection or non-resectable cancer had higher occult tumour cell positivity in peritoneal lavage than lower stage and grade patients (Table 2). We found a significant positive association ( $P<0.03$ ) between CEA transcript levels in peritoneal lavage and the clinical stage, i.e. levels were higher in samples from patients with advanced disease (Fig. 1A). We also found that EGFR transcript levels were significantly higher ( $P<0.04$ ) in peritoneal lavage samples from patients with high grade tumours than in samples from patients with low grade tumours (Fig. 2B). In addition, CEA transcript levels were significantly lower ( $P<0.001$ ) in peritoneal lavage samples from patients who underwent radical surgery (R0) than in samples from patients who underwent R1+R2 or non-resectional surgery (Fig. 3A).

### Occult tumour cells and survival rate

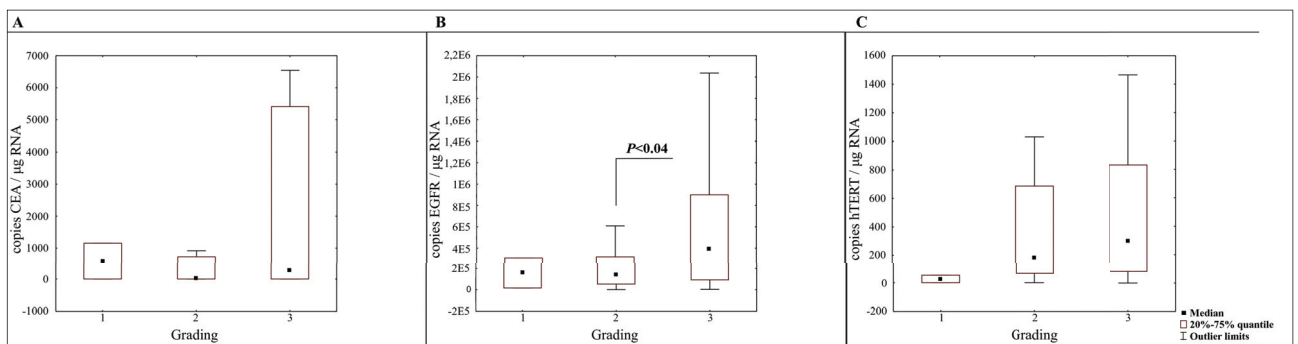
The patients who provided peritoneal lavage samples in which occult tumour cells were detected using the CEA (Fig. 4A), EGFR (Fig. 4B), CEA and/or EGFR (Fig. 4D) markers, and a combination of all three examined markers (Fig. 4E), had significantly shorter overall survival times ( $P<0.02$ ,  $P<0.01$ ,  $P<0.001$  and  $P<0.02$ ) and an increased hazard ratio for disease recurrence (HR=1.82 [95% CI: 1.08-3.07], HR=2.12 [95% CI: 1.15-3.93], HR=2.14 [95% CI: 1.30-3.55] and HR=1.62 [95% CI: 0.98-2.67]), than those who provided samples in which no occult tumour cells were detected. However, the presence/absence of occult tumour cells evaluated via expression of hTERT

**Table 2.** Correlation between occult tumor cell positivity (using CEA, EGFR and hTERT as markers) in peritoneal lavage and clinical-pathological features of pancreatic cancer patients.

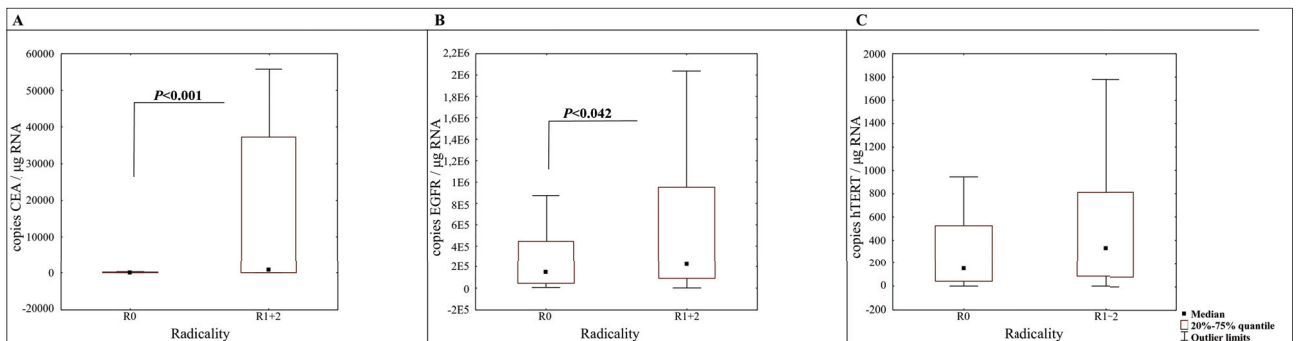
		Peritoneal lavage (positive/total (percentage))		
		CEA	EGFR	hTERT
Clinical Stage	1	0/3 (0)	1/3 (33.3)	2/3 (66.7)
	2	0/17 (0)	0/17 (0)	3/17 (17.7)
	3	3/21 (14.3)	1/21 (4.8)	5/21 (23.8)
	4	26/55 (47.3)	13/55 (23.6)	23/55 (41.8)
Grading	G1	1/2 (50.0)	0/2 (0)	0/2 (0)
	G2	11/50 (22.0)	6/50 (12.0)	15/50 (30.0)
	G3	15/40 (37.5)	8/40 (20.0)	16/40 (40.0)
Radicality	R0	4/42 (9.5)	2/42 (4.8)	11/42 (26.2)
	R1,2	25/54 (46.3)	13/54 (24.1)	22/54 (40.7)



**Fig. 1.** Correlation between levels of CEA (A), EGFR (B) and hTERT (C) mRNA as an occult tumor cell markers in the peritoneal lavage and clinical stage of pancreatic cancer patients.



**Fig. 2.** Correlation between levels of CEA (A), EGFR (B) and hTERT (C) mRNA in peritoneal lavage and tumor grades of pancreatic cancer patients.



**Fig. 3.** Correlation between levels of CEA (A), EGFR (B) and hTERT (C) mRNA in peritoneal lavage and surgical radicality of pancreatic cancer patients.

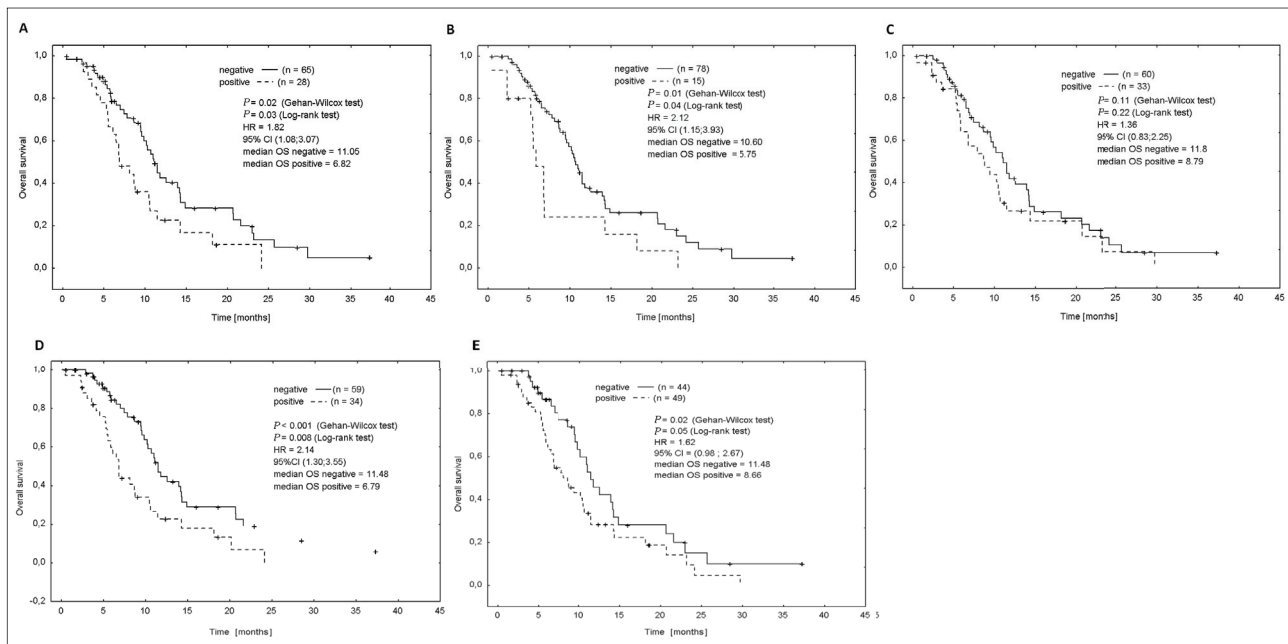
did not significantly associate with survival parameters (Fig. 4C), moreover the triple combination of CEA and/or EGFR and/or hTERT positivity showed weaker association with overall survival (Fig. 4E) than the CEA and/or EGFR alone (Fig. 4D), suggesting low value of hTERT in detection of occult tumour cells in pancreatic cancer.

## DISCUSSION

The aim of the study was to establish suitable tumour markers for detecting occult tumour cells in peritoneal lavage of patients with pancreatic cancer and to evaluate the clinical significance of those markers as prognostic fac-

tors. Several methods can be used to detect occult tumour cells. These, differ in time requirements, cost, specificity and, above all, sensitivity. Current molecular, PCR-based methods are the most sensitive; real time RT-PCR can detect as few as one occult tumour cell in  $1 \times 10^7$  cells, and is roughly two orders of magnitude more sensitive than immunocytochemical methods<sup>6-9</sup>. Thus it affords excellent sensitivity and, depending on the tumour marker used, specificity. Indeed, Schmidt et al. showed that CEA and AFP transcripts are highly specific markers of occult tumours in peritoneal lavage, detecting no elevated levels of either marker in lavage samples from patients with benign bowel disease<sup>10</sup>. Hence, CEA and AFP are potentially useful markers for specific early diagnosis of peritoneal dissemination of gastrointestinal





**Fig. 4.** Overall survival rates of pancreatic cancer patients in relation to the presence of occult tumor cells in peritoneal lavage, detected using RT-PCR amplification of CEA (A), EGFR (B), hTERT (C), combination of CEA and/or EGFR (D) or triple combination of CEA and/or EGFR and/or hTERT (E).

cancer. In contrast, cytokeratin 19 mRNA lacks specificity for gastrointestinal cancer. It should be noted that Broll et al. do not recommend PCR-based analysis of CEA transcripts for detecting occult tumour cells in peritoneal lavage because they found high false positive rates; five of 13 healthy control peritoneal lavage samples (38%) yielded positive results<sup>11</sup>. However, CEA positivity was detected in 63% of tested peritoneal lavage samples from pancreatic cancer patients (47/75) and PCR-positive patients showed in agreement with our observation poorer overall survival ( $P=0.04$ ).

To date, there have been few published PCR-based evaluations of the prognostic value of occult tumour cells in peritoneal lavage from pancreatic cancer patients. Hoffmann et al. detected occult tumour cells in peritoneal lavage samples from 30% (11/30) of tested pancreatic cancer patients using RT-PCR amplification of cytokeratin 19 (ref.<sup>12</sup>), finding that their presence correlated with tumour stage and differentiation ( $P<0.05$ ), and that cytokeratin-19 positive patients (weakly) tended to have shorter survival times than controls ( $P<0.15$ ). Eguchi et al. found CEA positivity, using PCR, in peritoneal lavage samples from 21.7% (15/69) of pancreatic cancer patients, and that these patients had inferior recurrence-free and overall survival rates ( $P=0.004$  and  $P=0.015$ ). Multivariate analysis also demonstrated that RT-PCR detection of CEA in peritoneal lavage was a significant prognostic factor (hazard ratio; 3.65). Sergeant et al. found a significant increase of PCR positivity for EpCAM in peritoneal lavage samples obtained postoperatively, relative to preoperative samples ( $P<0.0001$ ) (ref.<sup>14</sup>), but no significant association between EpCAM peritoneal lavage positivity and disease-free survival.

Thus, conflicting or ambiguous results have been obtained from previous analyses of occult tumour cells in peritoneal lavage samples from pancreatic cancer patients<sup>5,12-15</sup>. Major problems in this context are the differences in meth-

ods used and small sets of patients examined. In our study we evaluated a real-time RT-PCR method for CEA, EGFR and hTERT transcript analysis, which affords quantitative determination of specific marker expression and allows cut-off values to be set to overcome problems with false results. We also analysed a larger set of peritoneal lavage samples, from 96 pancreatic cancer patients, than previous authors. Patients with PCR positivity for CEA and EGFR in peritoneal lavage had higher clinical stages than others, and their tumours were poorly differentiated. Moreover, CEA- and EGFR-positive patients had significantly shorter overall survival times than negative patients. Thus, detection of occult tumour cells in peritoneal lavage of pancreatic cancer patients using PCR amplification of CEA and/or EGFR can identify patients with advanced disease and poor prognosis, for whom radical surgery is likely to have little benefit, hence it may potentially influence indications for radical surgery in the future.

## CONCLUSION

The present study demonstrates that the presence of occult tumour cells in peritoneal lavage is a negative prognostic factor for survival in pancreatic cancer patients. EGFR and CEA transcript levels in peritoneal lavage corresponded with the clinical stage, tumour grade and surgical radicality of the 96 patients. Identification of occult tumour cells using PCR-based methods is clinically feasible, provides reproducible results, and can identify patients with advanced disease for whom radical surgery is of little benefit. Further studies should focus on its applicability in the selection of pancreatic cancer patients, who may benefit from radical surgery, palliative surgery or conservative therapy only.

## ABBREVIATIONS

hTERT, Telomerase; EGFR1, Epidermal growth factor 1; CEA, Carcinoembryonic antigen; CK, Cytokeratin; AFP, alpha-fetoprotein.

## ACKNOWLEDGEMENTS

This study was supported by grants from the Internal Grant Agency of the Czech Ministry of Health (grant No. IGA NS 9937-4), Palacky University (IGA UP LF\_2012\_017), and the Ministry of Industry and Trade (MPO TIP FR-TI1/525). The infrastructural part of the project (the Institute of Molecular and Translational Medicine) was supported by the Operational Program Research and Development for Innovations (project CZ.1.05/2.1.00/01.0030).

## REFERENCES

1. Tani M, Kawai M, Terasawa H, Ina S, Hirono S, Shimamoto T, Miyazawa M, Uchiyama K, Yamaue H. Prognostic factors for long-term survival in patients with locally invasive pancreatic cancer. *J Hepatobiliary Pancreat Surg* 2007;14:545-50.
2. Wolff RA, Varadhachary GR, Evans DB. Adjuvant therapy for adenocarcinoma of the pancreas: analysis of reported trials and recommendations for future progress. *Ann Surg Oncol* 2008;2773-86.
3. Ryska M, Strnad R, Belina F, Zavoral M, Salek C, Hrabal P, Buric I, Laszikova E, Kvicerova H, Jurenka B, Holcatova I. Radical resection in patients with pancreatic head carcinomas. A retrospective survival analysis in a group of 307 subjects. *Rozhl Chir* 2007;86:432-9.
4. Lovecek M, Klos D, Skalicky P, Kysucan J, Neoral C, Melichar B, Havlik R. Surgery for cancer of head of pancreas and survival analysis. *Onkologie* 2010;4:338-41.
5. Klos D, Lovecek M, Srovnal J, Benedikova A, Ruzkova V, Radova L, Hajduch M, Neoral C, Havlik R. Possibility of using the determination of minimal residual disease in pancreatic adenocarcinoma using real-time RT-PCR – a pilot study. *Cas Lek Cesk* 2010;149:69-73.
6. Koch M, Kienle P, Hinz U, Antolovic D, Schmidt J, Herfarth C, Von Knebel Doerevitz M, Weitz J. Detection of hematogenous tumor cell dissemination predicts tumor relapse in patients undergoing surgical resection of colorectal liver metastases. *Ann Surg* 2005;241(2):206-7.
7. Niedergethmann M, Rexin M, Hildenbrand R, Knob S, Sturm JW, Richter A, Post S. Prognostic implications of routine, immunohistochemical, and molecular staging in resected pancreatic adenocarcinoma. *Am J Surg Pathol* 2002;26(12):1578-87.
8. Schuster R, Max N, Mann B, Heufelder K, Thilo F, Gröne J, Rokos F, Buhr HJ, Thiel E, Keiholz. Quantitative real-time RT-PCR for detection of disseminated tumor cells in blood of patients with colorectal cancer using different mRNA markers. *Int J Cancer* 2004;108:219-27.
9. Taniguchi T, Makino M, Suzuki K, Kaibara N. Prognostic significance of reverse transcriptase-polymerase chain reaction measurement of carcinoembryonic antigen mRNA levels in tumor drainage blood and peripheral blood of patients with colorectal carcinoma. *Am Cancer Soc* 2000;89(5):970-6.
10. Schmidt P, Thiele M, Rudroff C, Vaz A, Schilli M, Friedrich K, Scheele J. Detection of tumor cells in peritoneal lavages from patients with gastrointestinal cancer by multiplex reverse transcriptase PCR. *Hepatogastroenterology* 2001;48:1675-9.
11. Broll R, Weschta M, Windhoevel U, Berndt S, Schwandner O, Roblic U, Schiedeck THK, Schimmelpennig H, Bruch HP, Duchrow M. Prognostic significance of free gastrointestinal tumor cells in peritoneal lavage detected by immunocytochemistry and polymerase chain reaction. *Langenbecks Arch Surg* 2001;386(4):285-92.
12. Hoffmann K, Kerner C, Wilfert W, Mueller M, Thiery J, Hauss J, Witzigmann H. 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. *World J Gastroenterol* 2007;13:257-63.
13. Eguchi H, Ohigashi H, Takahashi H, Yano M, Motoori M, Miyashiro I, Kishi K, Ohue M, Noura S, Seki Y, Yamada T, Goto K, Yamamoto T, Idota A, Fujii J, Nakajima H, Yamasaki T, Ishikawa O. Presence of minute cancer cell dissemination in peritoneal lavage fluid detected by reverse transcription PCR is an independent prognostic factor in patients with resected pancreatic cancer. *Surgery* 2009;146:888-95.
14. Sergeant G, Roskams T, van Pelt J, Houtmeyers F, Aerts R, Topal B. Perioperative cancer cell dissemination detected with a real-time RT-PCR assay for EpCAM is not associated with worse prognosis in pancreatic ductal adenocarcinoma. *BMC Cancer* 2011;11:47.
15. Klos D, Lovecek M, Skalicky P, Srovnal J, Kesselova M, Radova L, Hajduch M, Neoral C, Havlik R. Minimal residual disease in pancreatic cancer – our first experiences. *Rozhl Chir* 2010;89:135-9.