Gene expression of cytokinesis regulators *PRC1*, *KIF14* and *CIT* has no prognostic role in colorectal and pancreatic cancer

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Abstract. Colorectal cancer is one of the most common cancers and pancreatic cancer is among the most fatal and difficult to treat. New prognostic biomarkers are urgently needed to improve the treatment of colorectal and pancreatic cancer. Protein regulating cytokinesis 1 (*PRC1*), kinesin family member 14 (*KIF14*) and citron Rho-interacting serine/threonine kinase (*CIT*) serve important roles in cytokinesis, are strongly associated with cancer progression and have prognostic potential. The present study aimed to investigate the prognostic relevance of the PRC1, KIF14 and CIT genes in colorectal and pancreatic cancer. *PRC1*, *KIF14* and *CIT* transcript expression was assessed by reverse transcription-quantitative PCR in tumors and paired distant unaffected mucosa from 67 patients with colorectal cancer and tumors and paired non-neoplastic control tissues from 48 patients with pancreatic cancer. The extent of transcript dysregulation between tumor and control tissues and between groups of patients divided by main clinical characteristics, namely patients' age and sex, disease stage, localization and grade, was determined. Finally, the associations of transcript levels in tumors with disease-free interval and overall survival time were evaluated. *PRC1*, *KIF14* and *CIT* transcripts were upregulated in tumors compared with control tissues. *PRC1*, *KIF14* and *CIT* levels strongly correlated to each other in both colorectal and pancreatic tumor and control tissues after correction for multiple testing. However, no significant associations were found among the transcript levels of *PRC1*, *KIF14* and *CIT* and disease-free interval or overall survival time. In summary, the present study demonstrated mutual correlation of PRC1, KIF14 and CIT cytokinesis regulators with no clear prognostic value in pancreatic and colorectal cancers. Hence, according to the results of the present study, transcript levels of these genes cannot be clinically exploited as prognostic biomarkers in colorectal or pancreatic cancer patients.

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Introduction

Colorectal cancer (CRC) is the second most frequent cancer in women and third most common in men worldwide (1) with more >1.9 million new cases reported in 2020 (2). CRC mortality rates are higher in developed countries and it was ranked second globally in the mortality ratings with >935,000 deaths caused by it in 2020 (2). CRC is usually diagnosed in later stages (regional or distant) which complicates the treatment and results in worse outcomes for patients (3).

Pancreatic ductal adenocarcinoma (PDAC) has a lower global incidence rate >495,000 compared with CRC (2), but with >466,000 deaths in 2020 worldwide (2) and the projected dramatic increase in incidence in the USA by 2030 (4), it remains one of the deadliest cancers. Late diagnosis, due to delayed manifestation of symptoms and generally very poor long-term response to systemic chemotherapy have complicated the treatment and resulted in worse outcomes for patients, hence these are now subject to state-of-the-art studies in the precision medicine field (5). Hence, the search for new diagnostic, prognostic and predictive biomarkers which will facilitate treatment at less advanced stages when outcomes are more favorable is necessary (6).

Cytokinesis is a key event that occurs at the end of cell division and is important for successful tissue proliferation (7).

One of its essential components is the constitution of a central mitotic spindle in which protein regulating cytokinesis 1 (PRC1), kinesin family member 14 (KIF14) and citron Rho-interacting serine/threonine kinase (CIT) proteins serve an important role (8). Mutations, defects and overall failures in cytokinesis may influence tumorigenesis in multiple tissues, e.g., breast, gut, lung, pancreas and ovary (9).

One of the most important components of cell division is PRC1, a substrate of several cyclin-dependent kinases, e.g., CDK1, CDK2 and CDK6, which helps to regulate their levels during the cell cycle (10). During interphase, PRC1 localizes almost exclusively to the cell nucleus (10), but when mitosis commences it redistributes and binds to microtubules maintaining the spindle midzone in early stages and to the midbody during cell cleavage (11). Reduction of PRC1 activity in cells prevents completion of cellular cleavage, but not nuclear division (11).

Multiple proteins of the kinesin-motor family depend on PRC1 and amongst others, KIF14, is one of them (12-14). KIF14 is a member of the kinesin-3 subfamily which exists in the cell as a dimer (15) and has ATPase activity (16). KIF14 localizes to the cytoplasm during interphase, but after the start of mitosis it accumulates at spindle poles (17). Later in anaphase, KIF14 becomes concentrated at the spindle midzone and midbody (17). Cells with depleted KIF14 fail to complete cytokinesis (17). KIF14 interacts with both PRC1 and the RhoA kinase regulator CIT (18,19). CIT is necessary for localization of KIF14 and important for cell division (7) and orientation of the mitotic spindle during metaphase (20).

It has been already reported in our earlier studies that expression levels of *PRC1*, *KIF14* and *CIT* are significantly elevated in tumor tissues, such as breast (21) and ovarian (22) carcinomas. In addition, patients with breast cancer with high *PRC1* expression level in tumors had significantly worse disease-free survival time compared with the rest of patients (21), and low *CIT* level was associated with worse time to progression in patients with ovarian cancer (22). In fact, the prognostic role of these regulators of cytokinesis was recently suggested by others in hepatocellular, prostate and bladder cancer (23-25).

The present study aimed to assess the prognostic relevance of the cytokinesis regulators in *PRC1*, *KIF14* and *CIT* in patients with CRC and PDAC. *PRC1*, *KIF14* and *CIT* were selected based on previous functional evidence and putative prognostic roles reported in other carcinomas (21-25).

Materials and methods

Experimental subjects. Tissue samples of primary tumors of human colorectal carcinoma and paired distant unaffected mucosa, where possible at least 20 cm from the primary tumor site, were collected from 67 patients (age range, 39-79 years old) with CRC diagnosed and treated at the Department of Surgery, Teaching Hospital in Pilsen (Pilsen, Czech Republic) during the period February 2008-August 2010 as described before (26). The following inclusion criteria were applied to the recruitment of patients into the study: i) Patients who were subject to surgery for CRC; ii) no prior chemotherapy before surgery (in order to eliminate its influence on transcript levels); iii) patients who received only first-line chemotherapy in either a palliative or adjuvant setting; and iv) patients who received adjuvant regimens based on 5-fluorouracil, leucovorin (de Gramont or FUFA), ftorafur, capecitabine and/or oxaliplatin (FOLFOX) or palliative chemotherapy based on FOLFOX regimen in combination with or without Avastin (n=3 untreated). Native tissue samples were taken during resection surgery, macrodissected, snap-frozen in liquid nitrogen and stored at -80˚C until total RNA isolation. The control mucosa samples were taken from the macroscopically unaffected resection margins of colon tissues. The resection margins were microscopically evaluated and only samples free of malignant cells were further analyzed. Corresponding tumor tissue samples were verified by an independent experienced pathologist (Teaching Hospital in Pilsen, Pilsen, Czech Republic). Only histologically-verified samples were included in this study. The following data on patients were retrieved from medical records during regular hospital-based follow-up: Age, sex, date of diagnosis, tumor localization, pathological tumor node metastasis (TNM stage) according to The Union for International Cancer Control (UICC) 6th Edition (27), histological type and grade of the tumor, adjuvant (group A) or first line of palliative (group B) chemotherapy, treatment response in group B patients and disease-free interval (DFI) and overall survival time (OS) in all patients (Table I). Patients were followed by imaging techniques and assessment of circulating tumor markers (CEA and CA 19-9) every 3 months during the first two years after adjuvant chemotherapy and then every 6 months for the next 3 years. Response to the treatment was evaluated by the Response Evaluation Criteria In Solid Tumors (RECIST) criteria version 1.0 (28) based on routinely used imaging techniques (by computer tomography, with or without positron emission tomography, magnetic resonance or ultrasonography) for assessment of tumor mass. Response to the treatment was defined as a decrease of the number or volume of metastases or stabilization of the disease. In patients treated with adjuvant therapy after radical surgical resection R0 (group A), DFI served as the treatment outcome for analyses. DFI was defined as the time elapsed between radical surgical R0 resection and disease recurrence.

A cohort of 48 patients (age range, 46-80 years old) with PDAC who underwent curative intent surgery between August 2009 and January 2012 were recruited from the Institute of Clinical and Experimental Medicine, Prague and the University Hospital (Brno, Czech Republic) as described before (29). The following inclusion criteria were applied to the recruitment of patients into the study: i) Patients who were subject to surgery for PDAC; ii) had no prior chemotherapy before surgery (in order to eliminate its influence on transcript levels); and iii) pathologically confirmed PDAC diagnosis. Patients treated with preoperative chemo (radio) therapy were excluded from the study. The resection specimens from these patients were immediately transferred from the operating theater to the Pathology Department, macrodissected to differentiate tumor and paired non-neoplastic (control) tissues and then snap-frozen in liquid nitrogen. The histologically verified samples of tumors and control tissues were then stored at -80˚C until RNA extraction. Histological diagnosis of PDAC was performed according to the standard classification. The clinical data including age, sex, date of diagnosis, tumor localization, pTNM stage, the histological type and grade of the tumor, resection margin status, lymphatic, vascular and perineural

Table I. Clinical characteristics of patients with CRC (n=67).

Characteristics	Value
Age at diagnosis mean ± SD, years	63.6±8.8
Sex, n (%)	
Female	23 (34)
Male	44 (66)
Primary tumor localization, n (%)	
Colon or sigma	42 (73)
Rectum or rectosigmoid junction	25 (27)
Primary tumor size, location and invasive depth (pT), n (%)	
pT2	5 (7)
pT3	53 (79)
pT4	9 (13)
Lymph node metastasis (pN), n (%)	
pN0	22 (33)
pN1	27 (40)
pN2	18 (27)
Distant metastasis (cM), n (%)	
cM0	34 (51)
cM1	33 (49)
Pathological stage (S), n (%)	
SII	14 (21)
SIII	20 (30)
SIV	33 (49)
Pathological grade (G), n (%)	
G1	10 (15)
G2	46 (69)
G3	11 (16)
Response to palliative chemotherapy in SIV patients[a]	
Regression or stabilization	15 (50)
Stable disease or progression	15 (50)
Not evaluated	3 (-)
Adjuvant chemotherapy in SII and SIII patients	
De Gramont or FUFA regimens	13 (38)
FOLFOX regimen	12 (35)
Capecitabine	8 (24)
Ftorafur	1 (3)

[a]Patients were treated with palliative chemotherapy based on FOLFOX regimen in combination with Avastin (n=16) or without (n=12). A total of 3 patients received de Gramont regimen and 2 received no palliative chemotherapy. CRC, colorectal cancer.

Table II. Clinical characteristics of patients with PDAC (n=48).

Characteristics	Value
Age at diagnosis mean ± SD, years	62.6±7.5
Sex, n (%)	
Female	26 (54)
Male	22 (46)
Primary tumor localization, n (%)	
Head	40 (83)
Body or tail	8 (17)
Primary tumor size, location, and invasive depth (pT), n (%)	
pT1	1 (2)
pT2	5 (10)
pT3	41 (86)
pT4	1 (2)
Lymph node metastasis (pN), n (%)	
pN0	19 (40)
pN1	29 (60)
Pathological stage (S), n (%)	
SI	3 (6)
SII	43 (90)
SIII	2 (4)
Pathological grade (G), n (%)	
G1	2 (4)
G2	29 (61)
G3	15 (31)
G4	2 (4)
Angioinvasion (pA), n (%)	
pA0	28 (58)
pA1	20 (42)
Perineural invasion (pP), n (%)	
pP0	11 (23)
pP1	37 (77)
Resection margins (R), n (%)	
R0	44 (92)
R1	4 (8)
Adjuvant chemotherapy, n (%)	
None	17 (35)
Gemcitabine or 5-fluorouracil based	31 (65)

PDAC, pancreatic adenocarcinoma.

invasion, adjuvant chemotherapy based on gemcitabine or 5-fluorouracil (n=17 untreated) and OS were all obtained from medical records during regular hospital-based follow-up (Table II).

Study protocol was approved by the Ethical Commission of the Medical Faculty and Teaching Hospital in Pilsen (Pilsen, Czech Republic) (approval no. IGA 10230-3, 2nd September 2008) and the Institutional Review Boards of the Institute of Clinical and Experimental Medicine (Prague, Czech Republic) and the University Hospital Brno (Brno, Czech Republic) (approval received in the process of application of research project. no. GA CR P304/10/0338, 4th May 2009). Written informed consent was obtained from all individual participants included in the study.

RNA isolation and cDNA preparation. Total RNA was isolated according to the procedure published elsewhere (30). Briefly,

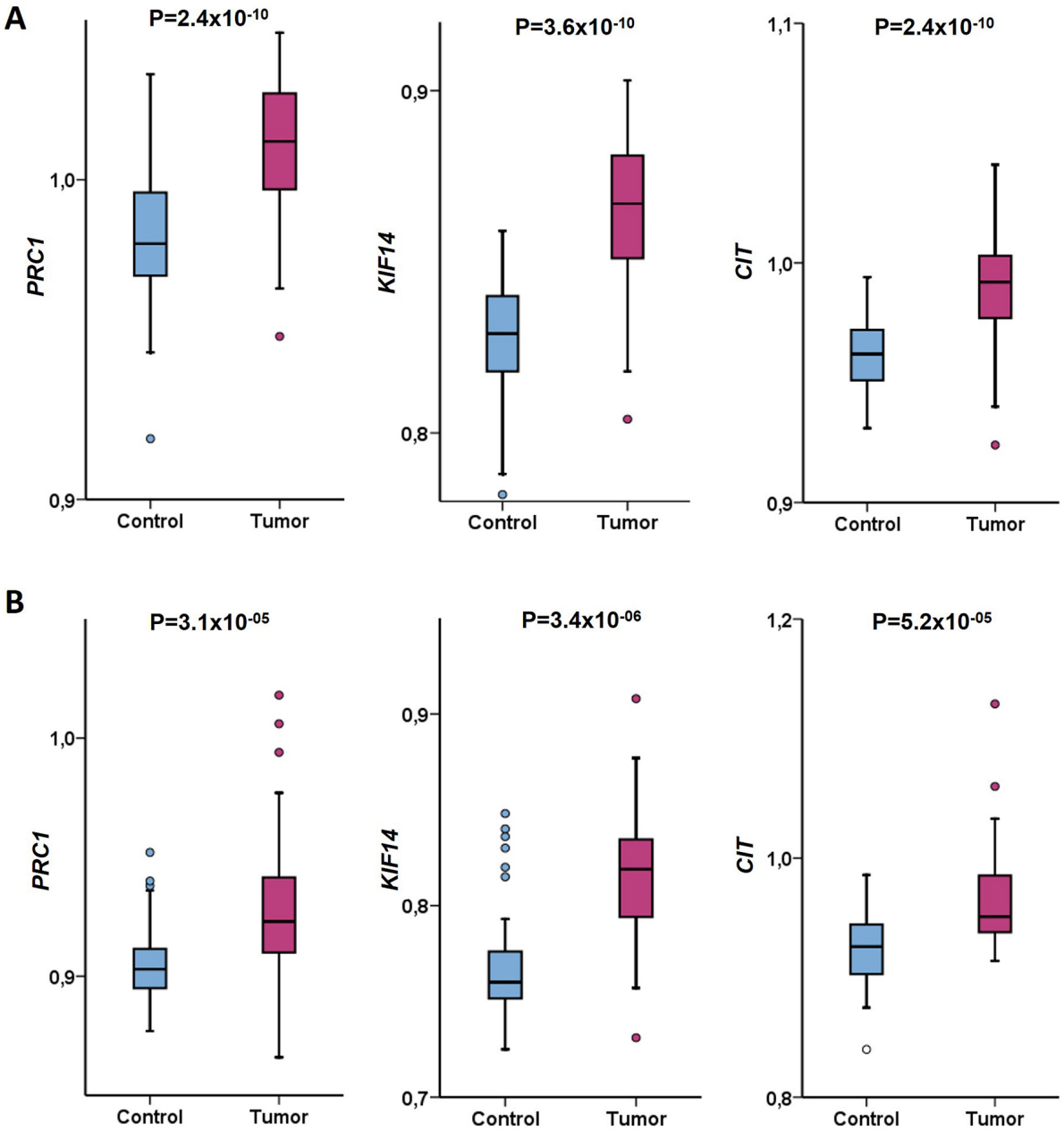

Figure 1. Box plot of gene expression distribution between tumor and control tissues of patients with (A) CRC (n=67) and (B) PDAC (n=48). Ct ratio which was an arithmetic mean of Ct for all reference genes to a particular target gene was calculated for each sample. Outliers are displayed as circles. PRC1, protein regulating cytokinesis 1; KIF 14, kinesin family member 14; CIT, citron Rho-interacting serine/threonine kinase; CRC, colorectal cancer; PDAC, pancreatic adenocarcinoma.

fresh frozen tumor and control tissues (~2x2x2 mm blocks) were first homogenized by mechanical disruption using a Precellys instrument (Bertin Technologies SAS; CNIM Group) at a speed of 6,500 rpm for 15 sec at room temperature. Total RNA was isolated from all samples using Trizol® (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's instructions and stored in 20 μl aliquots at -80˚C. The RNA quantity was assessed in duplicates by Quant-iT RiboGreen RNA Assay kit (Invitrogen; Thermo Fisher Scientific Inc.) using the Infinite M200 multimode reader (Tecan Group, Ltd.). The quality was assessed by measurement of RNA Integrity Number (RIN) using Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Assay Kit (Agilent Technologies, Inc.) and samples with RIN ≥3 were used for analysis. For cDNA synthesis, 0.5 μg of the isolated RNA was used with the RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc.). The reaction was incubated 60 min at 42˚C and terminated by heating at 70˚C for 5 min. PCR amplification of ubiquitin C discriminating between product from cDNA (190 bp) and from genomic DNA (1,009 bp) was used for the cDNA quality check in terms of DNA contamination as described before (31). All cDNA samples that were free of DNA contamination (absence of 1,009 bp band in sample incubated without reverse transcriptase) were further analyzed.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was performed using the LightCycler® 96 System

Table III. Clinical characteristics of patients with CRC (n=67) and expression of target genes (PRC1, KIF14 and CIT).

		PRC1	*KIF14*	*CIT*
Characteristics	n	P-value[a] (ϱ value)		
Age	67	0.484[b] (0.095)	0.125[b] (-0.207)	0.711[b] (0.051)
Sex		0.430	0.329	0.063
Female	23			
Male	44			
Primary tumor localization		0.280	0.275	0.370
Colon	28			
Sigma	13			
Rectum	15			
Primary tumor size, location, and invasive depth (pT)		0.554	0.273	0.308
pT2	5			
pT3	53			
pT4	9			
Lymph node metastasis (pN)		0.769	0.357	0.913
pN0	22			
pN1-2	45			
Distant metastasis (cM)		0.822	0.882	0.722
cM0	34			
cM1	33			
Pathological stage (S)		0.176	0.020[c]	0.066
SII	14			
SIII	20			
SIV	33			
Pathological grade (G)		0.810	0.453	0.452
G1	10			
G2	46			
G3	11			
Response to palliative chemotherapy		0.658	0.877	0.913
Regression or stable	15			
Progression	15			

[a]The Kruskal-Wallis test; [b]the Spearman correlation test, ϱ=correlation coefficient; [c]the Jonckheere-Terpstra trend test non-significant. PRC1, protein regulating cytokinesis 1; KIF 14, kinesin family member 14; CIT, citron Rho-interacting serine/threonine kinase; CRC, colorectal cancer.

(Roche Diagnostics GmbH). The reaction contained 2.5 μl of cDNA samples (diluted 10-times) and 7.5 μl of kit composed of 0.5 μl of TaqMan™ Gene Expression Assay (20X), 2 μl of 5X Hot FirePol Probe qPCR Mix Plus (ROX) and 5 μl of RNAse free water (all Thermo Fisher Scientific Inc.). The thermocycling conditions used were as follows: 50˚C for 2 min, denaturation at 95˚C for 10 min followed by 55 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 1 min. Mitochondrial ribosomal protein L19 (*MRPL19*), Polymerase II RNA subunit A *(POLR2A)* and Proteasome 26S subunit ATPase 4 (*PSMC4)* were used as reference genes specific for studies of human CRC and Eukaryotic translation initiation factor 2B *(EIF2B1)*, Eukaryotic translation initiation factor 1 (*ELF1)*, *MRPL19* and POP4 homolog, ribonuclease P/MRP subunit *(POP4)* for PDAC based on our previously published data (26,30). Primers and probes for RT-qPCR were part of commercially provided TaqMan™ Gene Expression Assays (Thermo Fisher Scientific Inc.). The list of genes and TaqMan™ Gene Expression Assays used in the study were listed in Table SI. The non-template control contained water instead of cDNA. Negative cDNA synthesis controls (RNA transcribed without reverse transcriptase) were also employed to demonstrate possible carry-over contamination. Each sample was assayed in duplicate and the mean value used for calculations. Samples with a variation >0.5 Ct (cycle threshold) were reanalyzed. The efficiencies of all assays were between 90 and 100% and calibration curves had $R^2 \geq 0.998$. Transcript levels were analyzed by Roche LightCycler® 96 System Software. Ratio of Ct of an arithmetic mean of Ct of all reference genes to a particular target gene was calculated

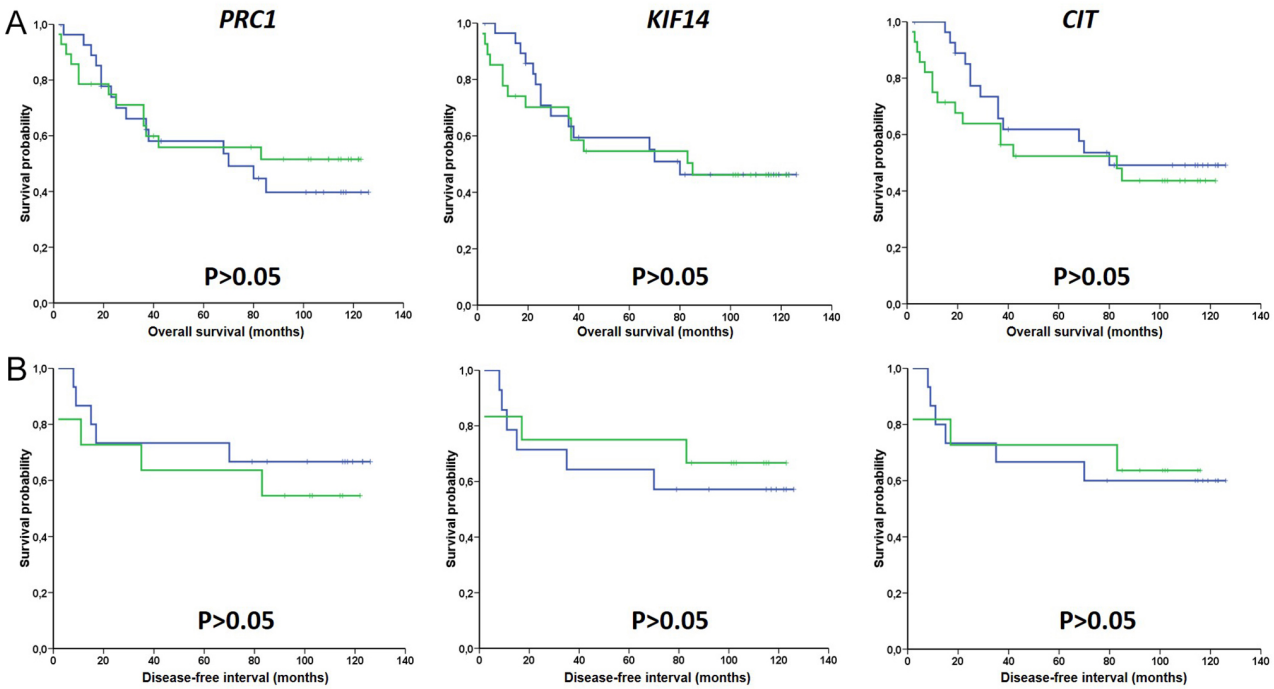

Figure 2. Associations between target gene expression and survival of patients with CRC. Kaplan-Meier survival curves were plotted for patients divided into groups above and below the median expression of *PRC1, KIF14* and *CIT*. The blue line represents the group with lower and green line the group with higher expression levels than median. (A) Overall survival time and (B) disease-free interval were presented. PRC1, protein regulating cytokinesis 1; KIF 14, kinesin family member 14; CIT, citron Rho-interacting serine/threonine kinase; CRC, colorectal cancer; PDAC, pancreatic adenocarcinoma.

for each sample and used for statistical evaluation (32). The qPCR study adhered to the MIQE Guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) (33).

Statistical analysis. Each sample was assayed in duplicate and the mean values were used for statistics. Ct ratio which was an arithmetic mean of Ct for all reference genes to a particular target gene was calculated for each sample. Gene expression levels are presented as mean ± SD (standard deviation) of this ratio (Fig. 1). Statistical analysis was performed using SPSS v.16.0 (SPSS, Inc.) as previously described (21,22). Briefly, distribution of gene expression data was evaluated by the Kolmogorov-Smirnov test. Gene expression data did not follow a normal distribution, and hence, non-parametric tests (the Wilcoxon signed-rank test, the Mann-Whitney test and the Kruskal-Wallis test) were used for evaluation of differences between groups of patients divided by the clinical data. Differences of gene expression levels in patients divided by sex, tumor localization and size, lymph node and distant metastasis, pathological stage and grade, resection margins and response to therapy were evaluated by Mann-Whitney and Kruskal-Wallis tests. The Spearman's rank correlation test was used for assessment of correlations between continuous variables as patient age and gene expression levels. Differences in gene expression levels between tumor and control samples were evaluated by the Wilcoxon signed-rank test. Receiver operator curve (ROC) analysis was performed for evaluation of the power of expression biomarkers to discriminate the patients with different survival functions. To divide the patients into low and high expressing groups, gene expression levels were divided by the median and cutoff values calculated using the ROC analysis performed using ROC Plotter (34). Median cutoff values were the following: 0.990, 1.160 and 1.020 for *PRC1, KIF14* and *CIT* DFI analysis in CRC tumors, respectively; 0.990, 1.157 and 1.015 for *PRC1, KIF14* and *CIT* OS analysis in CRC tumors, respectively and 1.083, 1.221 and 1.052 for *PRC1, KIF14* and *CIT* OS analysis in PDAC tumors, respectively. Cutoff values based on the ROC analysis were the following: 1.000, 1.170 and 1.020 for *PRC1, KIF14* and *CIT* OS analysis in CRC tumors, respectively and 1.084, 1.204 and 1.044 for *PRC1, KIF14* and *CIT* OS analysis in PDAC tumors, respectively. Survival function was plotted by the Kaplan-Meier method and the log rank test used for survival comparisons between groups of patients. To provide better estimates of survival probabilities and cumulative hazard, multivariate analysis was performed using Cox regression adjusted to stage. The correction for false discovery rate (FDR) was applied according to Benjamini and Hochberg (35). All P-values are from two-sided tests. $P<0.05$ was considered to indicate a statistically significant difference. Basic data about protein expression of PRC1, KIF14 and CIT in CRC and PDAC were extracted from The Human Protein Atlas (https://www.proteinatlas.org).

Results

Gene expression levels in tumor and control tissues of colorectal cancer patients. First, transcript expression levels of cytokinesis genes were assessed in tumor and control tissues from patients with CRC by RT-qPCR. *PRC1*, *KIF14* and *CIT* were significantly upregulated in tumors compared with paired control tissue samples (*PRC1*, $P=2.4x10^{-10}$; *KIF14*, $P=3.6x10^{-10}$; *CIT*, $P=2.4x10^{-10}$; Wilcoxon signed-rank test; Fig. 1A). *KIF14* and *CIT* expression was highly significantly

Table IV. Stage adjusted (A) overall survival and (B) disease-free interval of patients with CRC evaluated by Cox regression.

A, Overall survival of all patients (n=67)

Transcript	P-value	Hazard ratio[a]	95% confidence interval
PRC1	0.650	1.19	0.56-2.52
KIF14	0.889	1.06	0.49-2.28
CIT	0.920	0.96	0.42-2.17

B, Disease-free interval of patients treated with adjuvant chemotherapy (n=34)

Transcript	P-value	Hazard ratio[a]	95% confidence interval
PRC1	0.492	0.65	0.18-2.25
KIF14	0.729	1.26	0.34-4.70
CIT	0.898	0.91	0.20-4.03

[a]Hazard ratio, characterizing the chance for disease relapse or patient's death, for low expression level. CRC, colorectal cancer; PRC1, protein regulating cytokinesis 1; KIF 14, kinesin family member 14; CIT, citron Rho-interacting serine/threonine kinase; CRC, colorectal cancer.

correlated in tumor tissues ($P=3.0x10^{-09}$, correlation coefficient $\varrho=0.708$; Spearman correlation test) while correlations between *PRC1* and *KIF14* and *PRC1* and *CIT* were weaker ($P=4.2xE^{-05}$, $\varrho=0.532$ and $P=3.2xE^{-05}$, $\varrho=0.538$, respectively; Spearman correlation test). In control tissues, correlation between *PRC1* and *KIF14* was stronger ($P=3.7x10^{-07}$, $\varrho=0.633$; Spearman correlation test) compared with the rest of the correlations ($P=0.005$, $\varrho=0.378$ for *PRC1* with *CIT* and $P=2.1xE^{-04}$; $\varrho=0.487$ for *KIF14* with *CIT;* Spearman correlation test). All associations passed the FDR test for multiple testing ($q=0.016$). Data about protein expression from The Human Protein Atlas demonstrated that in general, PRC1 and KIF14 are overexpressed in colon or rectal carcinomas compared to normal tissues. On the other hand, CIT was downregulated in tumors (Table SII). The above results suggested that all cytokinesis genes were significantly upregulated in colorectal carcinomas compared to control tissues and that their levels mutually correlated.

Associations between gene expression levels in tumors and clinical data of patients with colorectal cancer. Then, *PRC1, KIF14* and *CIT* levels in tumor samples were compared in groups of patients stratified by clinical data and survival functions. No significant associations between intra-tumoral expression of *PRC1, KIF14* and *CIT* and age, sex, primary tumor size, location and invasive depth (pT), regional lymph node involvement (pN), distant metastasis (cM), grade or localization of tumor (colon, sigma, or rectum) were observed (Table III). *KIF14* expression significantly differed between patients stratified by the disease stage ($P=0.020$; Kruskal-Wallis test; Table III). However, the lack of trend between all the compared stages suggested that this association was not clinically relevant (Table III). In addition, it did not pass the FDR correction for multiple testing ($q=0.002$). No association between gene expression and response of patients to palliative therapy (progressive disease vs partial response or stable disease, group B, n=30) was found (Table III). Similarly, transcript expression levels of *PRC1, KIF14* and *CIT* did not associate with DFI of subgroup of patients treated with adjuvant chemotherapy (group A, n=34) or with OS of all patients (both groups together) as evaluated by the gene expression median (Fig. 2) or cutoff using the ROC analysis (Fig. S1, for OS only). Multivariate analysis by Cox regression adjusted to disease stage also failed to show significant associations between intra-tumoral expression levels of *PRC1, KIF14* and *CIT* and survival of patients with CRC (Table IV). Hence, gene expression levels were not associated with survival time in both univariate and multivariate analyses. Taken together, these analyses suggested a lack of significant associations between intra-tumoral expression of cytokinesis genes and clinical data of patients with CRC. Transcript expression of these genes was not associated with prognosis of patients assessed by DFI and OS.

Gene expression levels in tumors and control tissues of patients with pancreatic cancer. Expression levels of cytokinesis genes were assessed in tumor and control tissues from patients with PDAC by RT-qPCR. *PRC1*, *KIF14* and *CIT* were significantly upregulated in tumors compared with paired control tissue samples (*PRC1*, $P=3.1x10^{-05}$; *KIF14*, $P=3.4x10^{-06}$; *CIT*, $P=5.2x10^{-05}$; Wilcoxon signed-rank test; Fig. 1B). *PRC1* and *KIF14* expression was highly significantly correlated in tumor tissues ($P=1.1x10^{-11}$, $\varrho=0.852$; Spearman correlation test) while correlations between *KIF14* and *CIT* and *PRC1* and *CIT* were weaker ($P=0.004$, $\varrho=0.456$ and $P=0.001$, $\varrho=0.513$, respectively; Spearman correlation test). All genes correlated together in the same way in control tissues. However, correlations were of lower significance ($P=8.5x10^{-04}$, $\varrho=0.519$ for *PRC1* with *KIF14*; $P=0.004$, $\varrho=0.461$, for *PRC1* with *CIT* and $P=0.046$, $\varrho=0.326$, for *KIF14* with *CIT*; Spearman correlation test) compared with tumors. All associations except correlation of *KIF14* with *CIT* in control tissues passed the FDR test for multiple testing ($q=0.016$). Data about protein expression from The Human Protein Atlas demonstrated that in general, PRC1,

Table V. Clinical characteristics of patients with PDAC (n=48) and expression of target genes (PRC1, KIF14 and CIT).

		PRC1	*KIF14*	*CIT*
Characteristics	n	P-value[a] (ϱ value)		
Age at diagnosis	48	0.605[b] (0.076)	0.673[b] (0.063)	0.282[b] (0.158)
Sex		0.251	0.214	0.796
Female	26			
Male	22			
Primary tumor localization		0.463	0.354	0.154
Head	40			
Body or tail	8			
Primary tumor size, location, and invasive depth (pT)		0.852	0.743	0.105
pT1 or pT2	6			
pT3 or pT4	42			
Lymph node metastasis (pN)		0.480	0.650	0.704
pN0	19			
pN1	29			
Pathological stage (S)		0.180	0.418	0.120
SI	3			
SII or SIII	45			
Pathological grade (G)		0.690	0.714	0.126
G1 or G2	31			
G3 or G4	17			
Angioinvasion (pA)		0.917	0.842	0.975
pA0	28			
pA1	20			
Perineural invasion (pP)		0.056	0.079	0.084
pP0	11			
pP1	37			
Resection margins (R)		0.401	0.794	0.867
R0	44			
R1	4			

[a]The Kruskal-Wallis test; [b]the Spearman correlation test, ϱ=correlation coefficient. PRC1, protein regulating cytokinesis 1; KIF 14, kinesin family member 14; CIT, citron Rho-interacting serine/threonine kinase; PDAC, pancreatic adenocarcinoma.

KIF14 and CIT were overexpressed in pancreatic carcinomas compared to normal tissues (Table SII). The above analyses suggested that all cytokinesis genes were significantly upregulated in pancreatic carcinomas compared with control tissues and that their levels mutually correlated.

Associations between gene expression levels in tumors and clinical data of patients with pancreatic cancer. PRC1, KIF14 and *CIT* levels in tumor samples were compared in groups of patients stratified by clinical data and survival functions. No significant associations between intra-tumoral expression of *PRC1, KIF14* and *CIT* and individual clinical data of patients age, sex, tumor localization (head versus body or tail), pT, pN, stage, grade, angioinvasion and perineural invasion or resection margins (R0 versus R1) were observed (Table V). Expression of *PRC1* or *KIF14* did not associate with OS of all patients as evaluated by the gene expression median (Fig. S2A) or cutoff using the ROC analysis (Fig. S3). Notably, patients with lower expression of *CIT* had significantly worse OS compared with patients with higher expression level (P<0.05 for median and P<0.05 for the ROC analysis cut offs; log rank test; Fig. 3), but this result did not pass the FDR test (q=0.016). The OS of the subgroup of patients with PDAC treated with adjuvant chemotherapy (n=31) was not significantly associated with expression of *PRC1, KIF14* and *CIT* (Fig. S2B). No significant association of expression levels with OS was observed in multivariate analyses using the Cox regression adjusted to stage (Table VI). Hence, gene expression levels were not associated with survival time in both univariate and multivariate analyses. Taken together, these analyses suggested a lack of significant associations between intra-tumoral expression of cytokinesis genes and clinical data of patients with PDAC. Transcript expression of these genes were not associated with prognosis of patients assessed by OS.

Table VI. Stage adjusted overall survival of patients with PDAC (n=48) evaluated by Cox regression.

Transcript	P-value	Hazard ratio[a]	95% confidence interval
PRC1	0.223	1.48	0.79-2.77
KIF14	0.507	1.24	0.66-2.31
CIT	0.126	1.66	0.87-3.15

[a]Hazard ratio, characterizing the chance for patient's death, for low expression level. PRC1, protein regulating cytokinesis 1; KIF 14, kinesin family member 14; CIT, citron Rho-interacting serine/threonine kinase; PDAC, pancreatic adenocarcinoma.

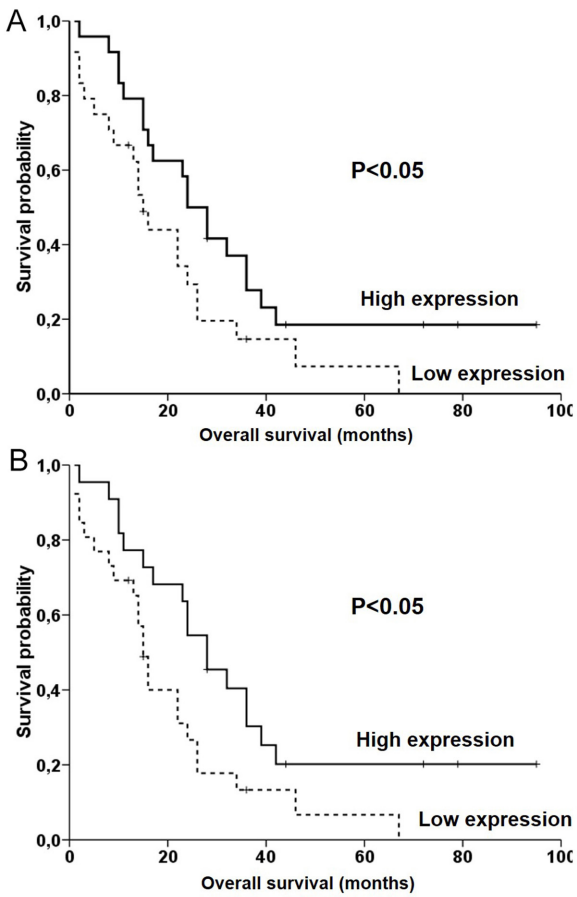

Figure 3. Association between expression of *CIT* and overall survival of patients with PDAC. Kaplan-Meier survival curves were plotted for the overall survival time of patients divided into the groups above and below (A) median expression of *CIT* or (B) according to the ROC analysis. Dashed line represents the group with lower expression and solid line the group with higher expression levels than median. CIT, citron Rho-interacting serine/threonine kinase; PDAC, pancreatic adenocarcinoma; ROC, receiver operating curve.

Discussion

The present study focused on exploring whether transcript expression levels of *PRC1*, *KIF14* and *CIT* correlate with prognosis of patients with colorectal and pancreatic carcinomas. Although significantly increased gene expression levels of all 3 genes in both types of carcinomas compared with control tissues was observed in the present study, no association of *PRC1*, *KIF14* and *CIT* levels with DFI or OS in any of the patient groups studied was observed.

PRC1, *KIF14* and *CIT* transcript expression is dysregulated, mainly upregulated, in several malignancies, e.g., breast, hepatocellular, bladder, prostate and ovarian cancers (21-25) and therefore, the relevance of these cytokinesis regulators for tumor initiation and progression is being intensively studied currently (36-38). Transcript levels of all 3 genes strongly correlated together in the present study further demonstrating their importance for cancer progression and potential as potential therapeutic targets.

Prognostic significance of *PRC1*, *KIF14* and *CIT* has been so far reported in lung, ovarian, breast, cervical, gastric, hepatocellular, bladder and prostate cancers (21-25,39-45), however, the present study demonstrated no prognostic significance of *PRC1*, *KIF14* and *CIT* for CRC and PDAC.

Strong upregulation of *KIF14* transcript in tumors compared with control tissues has been previously observed in CRC in accordance with the results of the present study, however survival analysis was not investigated (46). Another study on CRC reported that CIT protein was upregulated in tumors compared with control tissues and disease-free and overall survival time were significantly poorer in patients with positive CIT protein staining in tumor tissues compared with patients with negative staining (47). In contrast, the present study observed poorer OS in patients with CRC with low intra-tumoral *CIT* transcript levels compared with patients with high levels, although this association did not pass the FDR test. Similarly, our earlier work demonstrated that low *CIT* transcript level is a negative prognostic factor in patients with ovarian carcinoma (22). According to *in vitro* studies, CIT functions in the late phase of cell division, early stage of mitosis and is responsible for DNA damage control (36-38). Dysregulation of CIT in several types of *in vitro* tumor models, e.g., breast, colorectal or cervical ones, leads to aneuploidy and chromosomal instability (CIN) (37). CIN may have various effects on the prognosis of patients, especially depending on the type of cancer and treatment, involving, e.g., genomic plasticity, inflammatory signaling, distant metastasis, immune evasion or resistance to therapy (48,49). The association of the degree of CIN and the expression of cytokinesis regulators with the clinical outcome of patients could be among the reasons for the observed discrepancies in patients with cancer. The difference between biological meaning of transcript levels and protein expression intensity represents other factor potentially explaining inconsistencies. For comparison the present study provided basic data about protein expression of *PRC1*, *KIF14* and *CIT* in CRC and PDAC extracted from The Human Protein Atlas (https://www.proteinatlas.org). These data demonstrated that most of the investigated cytokinesis genes (except CIT in CRC) were upregulated in CRC and PDAC compared with normal tissues also on the protein level.

Besides a few rather small-scale experimental studies in various cancer types, e.g., breast (21), ovarian (22,40), cervical (41) and prostate (25) carcinomas, Big Data mining, from the Gene Expression Omnibus (GEO) or the Cancer Genome Atlas (TCGA) databases, using various *in silico*

bioinformatics have demonstrated that some cytokinesis regulators may have prognostic value alone or as a part of a specific gene sets (50-52). For example, *KIF14* is one of 10 genes whose low transcript expression in the GSE62452 microarray dataset, composed of 69 PDAC tumors predicted significantly poorer OS time of patients with PDAC and this was also observed in the TCGA RNASeq dataset (50). More recently, *KIF14* was reported among 7 metastasis-related genes with prognostic potential based on OS time in PDAC based on 141 patients from the TCGA dataset validated with the GSE62452 dataset (51). Additionally, *PRC1* is among 10 other genes whose signature predicts OS time and 12 genes predicting disease-free survival time of patients with PDAC based on *in silico* analysis of data of 77 patients with PDAC from 3 GEO datasets (52). High intra-tumoral *CIT* transcript level alone was recently identified as poor prognosis predictor (both OS and disease-free survival time) in PDAC through analysis of 178 patients from the TCGA dataset using the Gene Expression Profiling Interactive Analysis online tool (53). In light of a recent study that almost 20% of samples in the TCGA dataset corresponded to normal or other than PDAC tissues and mainly that vast majority of genes lost prognostic significance after cohort curation (54), caution in performing *in silico* studies and mainly continuous verification of data in well characterized sample sets is necessary. The present study provided results acquired by analyzing cohorts of patients recruited in single center setting, hence reducing heterogeneity in clinical data reporting. Sample collection proceeded using long-term established logistics with minimum time-lapse between surgical specimen removal and processing including storage at low temperature in the present study. Sample processing, RT-qPCR analysis and evaluation of results are also standardized and maintained over long period (26,29,30). The control of these conditions in multicenter settings is very difficult if at all possible (55) suggesting that the exploitation of prognostic information in future precision medicine will rely on both single center and multicenter approaches.

The present study had several limitations. Modest sample size of the present study was a limitation. This was unavoidable considering 15-20% resection rate of PDAC (56). Additionally, archival formalin fixed paraffin embedded tissue samples are not suitable for analysis due to the poor RNA integrity. The study of transcript levels rather than protein expression is another limitation of the present study. The clinical relevance of protein analysis is obvious, i.e., it is routinely used for assessment of receptors or markers of proliferation. On the other hand, semi-quantitative immunohistochemistry reflects just protein level and not enzymatic activity (57). The issue of availability, specificity and selectivity of antibodies, variations in normalization of results and complexity of gene expression regulation frequently cause a lack of association between mRNA and protein levels and hence, they should be considered as independent markers (58). Therefore, separate future studies should investigate the roles of protein levels and potentially functional aspects of cytokinesis in PDAC and CRC.

In conclusion, the present study demonstrated upregulation and strong association of transcript levels of major cytokinesis regulators *PRC1*, *KIF14* and *CIT* in tumors from patients with CRC and PDAC without relevant prognostic significance. Hence according to the results of the present study, transcript levels of these genes cannot be clinically exploited as prognostic biomarkers in patients with colorectal or pancreatic cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PS, VL, MO and ZK were responsible for the general supervision of the study. PS and VB were responsible for study conceptualization. PS, VH, IK, and VB were responsible for experimental data analysis and interpretation. JR, RP, VL, MO, ZK, and BD were responsible for acquisition of clinical data. PS and VB drafted the manuscript. VH, VB, JR, RP, VL, MO, ZK, BD, IK and PS revised the manuscript for important intellectual content. PS and VL confirmed the authenticity of all the raw data. All authors have read and approved the manuscript and agreed to be accountable for all aspects of the work.

Ethics approval and consent to participate

The present study conformed with the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964). Study protocol was approved by the Ethical Commission of the Medical Faculty and Teaching Hospital in Pilsen (Pilsen, Czech Republic) (approval. no. IGA 10230-3, 2nd September 2008), the Institutional Review Boards of the Institute of Clinical and Experimental Medicine (Prague, Czech Republic) and the University Hospital Brno (Brno, Czech Republic) (approval received in the process of application of research project. no. GA CR P304/10/0338, 4th May 2009). Written informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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