

Increased detection rate and potential prognostic impact of disseminated tumor cells in patients undergoing endorectal ultrasound for rectal cancer

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Abstract

Background Hematogenous tumor-cell dissemination during diagnostic and therapeutic procedures in patients with colorectal cancer has been demonstrated.

Objective The aim of this study was to investigate the extent of disseminated tumor cells in blood samples of rectal cancer patients after endorectal ultrasound and to determine its prognostic relevance.

Materials and methods Peripheral venous blood samples from 45 patients with rectal cancer were taken before and after endorectal ultrasound. Blood samples were examined using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay to amplify cytokeratin 20 transcripts. Overall survival of the patients was calculated by the Kaplan–Meier method.

Results Disseminated tumor cells were detected in peripheral blood samples of 17 of 45 (38%) patients before and after endorectal ultrasound. Circulating tumor cells were found in 11 of 45 (24%) patients only after endorectal ultrasound ($p=0.006$). There was a clear trend toward a worse prognosis in patients with tumor cells in blood samples after endorectal ultrasound, but this difference was not statistically significant.

Conclusion This study demonstrates significantly increased hematogenous tumor-cell dissemination after endorectal

ultrasound in rectal cancer patients. Patients with tumor cells in blood samples after endorectal ultrasound tend to have a worse prognosis. The potential prognostic impact of this finding is presently unclear and has to be further validated in larger clinical trials.

Keywords Cytokeratin 20 · Disseminated tumor cells · Endorectal ultrasound · Rectal cancer

Introduction

Preoperative imaging and staging are the bases for an adequate therapy for patients with rectal cancer [1, 2]. Depending on tumor stage and localization, patients with rectal cancer are selected for different therapeutic strategies, e.g., preoperative chemotherapy and/or radiation in advanced tumor stages or solely surgical resection with total mesorectal excision in earlier tumor stages [3].

Although there are a variety of diagnostic modalities (digital examination, endorectal ultrasound, computed tomography, and magnetic resonance imaging) for preoperative local staging of rectal cancer, it is still controversial which of these methods has the highest accuracy. A metaanalysis comparing endorectal ultrasound, computed tomography, and magnetic resonance imaging for local staging and assessment of lymph node involvement in rectal cancer concluded that endorectal ultrasound was the best method for determining local tumor invasion [4]. For assessment of other prognostic factors such as lymph node involvement or adjacent organ invasion, none of the examined diagnostic procedures was found to be more accurate than the other [4].

There is clinical evidence that mechanical manipulation of malignant tumors contributes to hematogenous tumor-

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cell spread [5–11]. No study has yet investigated whether endorectal ultrasound as an intraluminal procedure induces hematogenous tumor-cell dissemination in rectal cancer due to mechanical alteration of the tumor.

On the basis of new molecular detection methods, e.g., polymerase chain reaction (PCR) and immunocytochemistry, the extent of tumor-cell dissemination in gastrointestinal cancers has been investigated more extensively during the last decade [12]. Sensitive and specific reverse transcriptase-PCR (RT-PCR)-based protocols have been developed which are able to detect one malignant cell in 10^7 normal peripheral mononuclear blood cells [13]. To determine the incidence and prognostic significance of hematogenous tumor-cell spread in colorectal cancer patients undergoing therapeutic and diagnostic procedures, these detection systems are now widely used [14–20]. Recently, we demonstrated significantly enhanced hematogenous tumor-cell dissemination during colonoscopy in patients with colorectal cancer [21].

The aim of this study was to examine the frequency and potential prognostic significance of hematogenous tumor-cell dissemination in patients with rectal cancer undergoing endorectal ultrasound.

Materials and methods

Patients

Patients were prospectively enrolled into this study between June 1999 and October 2000. Patients with histologically confirmed primary or recurrent rectal cancer undergoing subsequent surgical resection of the tumor with or without preoperative chemoradiation at the Department of Surgery, University of Heidelberg were eligible for this study. Informed consent was obtained from all patients. The study protocol was approved by the ethics committee of the University of Heidelberg. Participation did not delay treatment, and no patient underwent endorectal ultrasound for study reasons alone. Patients with other malignant diseases in their medical history were excluded. Tumor stage and grading were classified according to the fifth edition of the TNM classification of the International Union Against Cancer (UICC) [22].

Control group

Blood samples from two different groups of individuals served as controls. The first group comprised of 60 healthy volunteers, and from each person, a peripheral venous blood sample was taken. The second group consisted of nine patients with benign gastrointestinal diseases (e.g., rectal adenoma, Crohn's disease, and ulcerative colitis)

undergoing endorectal ultrasound. To exclude the possibility of the presence of exfoliated CK 20 expressing normal rectal mucosa cells in the peripheral circulation during endorectal ultrasound, blood samples from the second group were obtained before and after the procedure.

Defecation group

To determine whether colorectal cancer cells are shed into the circulation due to enhanced intraluminal pressure in the bowel, peripheral blood samples of ten patients with colorectal cancer were taken before and immediately after defecation.

Endorectal ultrasound

Endorectal ultrasound was done with a rigid endosonographic probe with a rotating 7 or 10 MHz scanner (Brüel und Kjær, Type 1846, Brüel u. Kjær Instruments, Marlborough, MA, USA) that generates a 360° image of the rectal wall. The examination was performed in the lithotomy position after cleansing the rectum with an enema in a standardized fashion. In short, the endosonography device was generally blindly advanced beyond the tumor and then slowly withdrawn while scanning. In cases of higher grade stenosis, a rigid rectoscope was inserted and used to visualize the lumen, thus facilitating the advancement of the endosonography device through the rectoscope. Acoustic coupling was achieved with the help of a water-filled balloon placed over the transducer. The required time for an endorectal ultrasound examination was between 10 and 15 min. In this study, endorectal ultrasound was performed by a single surgeon who is very experienced in this diagnostic procedure (>500 performed procedures). Biopsies from the tumor were not taken during the examination as all patients undergoing endorectal ultrasound already had histopathological confirmation of rectal cancer.

Blood sampling

A peripheral venous catheter was placed in a cubital vein and irrigated with 20 ml of normal saline to prevent contamination of the blood samples with skin cells. Before taking each blood sample, 10 ml of blood was drawn and discarded. Two blood samples (10 ml each) were obtained from each patient: the first immediately before endorectal ultrasound, the second in the first minute after the procedure. The blood samples were diluted with 10 ml phosphate-buffered saline (PBS). After density centrifugation (30 min, 400 g) through Ficoll-Paque (Pharmacia), mononuclear peripheral blood cells were harvested from the interphase and washed twice in PBS. The cell pellet was then shock-frozen in liquid nitrogen and stored at -70°C .

CK 20 RT-PCR

RNA extraction from peripheral mononuclear blood cells and CK 20 RT-PCR were performed as previously described [16]. PCR products were analyzed by electrophoresis on 2% agarose gels. PCR products were blotted onto nylon membranes (Hybond N+; Amersham Life Science, Buckinghamshire, U.K.) and hybridized with a chemoluminescence-labeled oligonucleotide probe as previously described [23]. The sensitivity of the CK 20 RT-PCR assay was determined in previous cell spiking experiments, allowing the detection of 10 HT 29 cells in 10 ml blood [16].

RNA quality and performance of reverse transcription of all analyzed samples was confirmed by RT-PCR amplification of glyceraldehyde phosphate dehydrogenase (GAPDH) transcripts as previously described [16].

Follow-up and statistical analysis

All patients were primarily included into the follow-up, but two patients were lost to follow-up after 12 months. The time of follow-up was calculated from the date of surgery. The survival was estimated according to the Kaplan–Meier method and compared using the log-rank test [24].

Tumor-cell detection in blood before vs after endorectal ultrasound was compared with the McNemar's test [25, 26]. The likelihood of tumor-cell dissemination during endorectal ultrasound in relation to complete advancement of the endosonography probe across the tumor was analyzed using Fisher's exact test. The correlation between tumor-cell dissemination and tumor height was computed with the chi-square test. Statistical significance was defined as $p \leq 0.05$. Statistical computations were done using the software package JMP (JMP, Cary, NC, USA).

Results

Control group

All blood samples from the 60 healthy volunteers and all blood samples from the nine patients taken before and after endorectal ultrasound for benign gastrointestinal diseases (e.g., rectal adenoma, Crohn's disease, and ulcerative colitis) consistently tested negative for CK 20 expression. The GAPDH RT-PCR was positive in every sample confirming adequate RNA quality and reverse transcription.

Defecation group

Five of the ten patients with colorectal cancer tested negative in both blood samples taken before and after defecation. In three patients, tumor cells were detected in

blood samples before and after defecation, and in two patients only in blood samples before defecation that tumor cells were detected. No case with tumor-cell detection only after defecation was observed.

Study group

Forty-five patients undergoing endorectal ultrasound for primary or recurrent rectal cancer at the Department of Surgery, University of Heidelberg (26 men, 19 women; median age 65) were included in this study. Five patients had recurrent rectal cancer (only intraluminal tumor recurrences), and 40 patients had primary rectal cancer. In six patients, only partial endorectal ultrasound could be performed due to tumor obstruction. Ten patients (4 patients with recurrent and 6 patients with primary rectal cancer) received preoperative chemoradiation before undergoing surgery. In these cases, endorectal ultrasound and blood sampling were performed before neoadjuvant therapy. In all patients, the presence of rectal adenocarcinoma was confirmed histopathologically.

Tumor cells were detected in at least one of the two blood samples taken before and after endorectal ultrasound in 29 of 45 patients (64%) with rectal cancer. In 17 of 45 patients (38%), CK 20 transcripts were detected before and after endorectal ultrasound, and in 1 of 45 patients (2%), tumor cells were found only in the blood sample taken before endorectal ultrasound. In 11 of 45 patients (24%), tumor cells were only detected after endorectal ultrasound (Table 1). Statistical analysis showed a significantly higher tumor-cell detection rate after endorectal ultrasound compared to the detection rate before endorectal ultrasound ($p=0.006$; McNemar's test). The significantly increased detection rate of tumor cells in blood samples taken after endorectal ultrasound was also shown when looking only at the 40 patients with primary rectal cancer ($p=0.012$; Table 2). Tumor-cell detection in blood samples after endorectal ultrasound was also increased in patients with advanced tumor stage of the rectal cancer. For the 40 patients with primary rectal cancer, the correlation between

Table 1 Correlation of tumor-cell detection in blood with timing of blood collection in 45 patients with primary and recurrent rectal cancer having undergone endorectal ultrasound

Before endorectal ultrasound	After endorectal ultrasound	Number of patients
Negative	Negative	16/45 (36%)
Positive	Positive	17/45 (38%)
Negative	Positive	11/45 (24%)*
Positive	Negative	1/45 (2%)
18/45 positive, 40%	28/45 positive, 62%*	

* $p=0.006$; McNemar's test

Table 2 Correlation of tumor-cell detection in blood with timing of blood collection in 40 patients with primary rectal cancer having undergone endorectal ultrasound

Before endorectal ultrasound	After endorectal ultrasound	Number of patients
Negative	Negative	15/40 (38%)
Positive	Positive	14/40 (35%)
Negative	Positive	10/40 (25%)*
Positive	Negative	1/40 (2%)
15/40 positive, 38%		24/40 positive, 60%*

* $p=0.012$; McNemar's test

tumor-cell detection including time point of blood collection and tumor stage is depicted in Table 3. The results were further analyzed to examine whether patients with obstructing tumors had an increased risk of mechanically induced tumor-cell dissemination compared to patients without tumor obstruction (Table 4). There was no difference in tumor-cell detection after endorectal ultrasound in the group of patients having undergone partial endorectal ultrasound compared to the group having undergone complete endorectal ultrasound.

As there are two main pathways of tumor-cell drainage via blood vessels in rectal cancer (along the iliac vein for tumors of the lower rectum and along the inferior mesenteric and portal vein for tumors of the middle and upper rectum), tumor-cell dissemination was correlated with the tumor height. There was no difference in tumor location when comparing tumor-cell-positive and tumor-cell-negative patients: the median height of tumors in the tumor-cell-positive patient group was 6.5 cm compared to 6 cm in the tumor-cell-negative group ($p=0.9$; chi-square test).

Follow-up

The median follow-up period for all patients was 51 months. No patient died because of tumor-unrelated reasons. The 5-year overall survival rate of patients with tumor cells in

Table 3 Correlation of tumor-cell detection in blood with timing of blood collection and tumor stage ($n=40$ patients with primary rectal cancer)

Stage	Tumor-cell detection			
	Before endorectal ultrasound		After endorectal ultrasound	
	Number of patients	Percentage	Number of patients	Percentage
I	5/15	33	8/15	53
II	2/7	29	3/7	43
III	5/15	33	10/15	75
IV	3/3	100	3/3	100

Table 4 Stratified analysis of patients with tumor cells detected only after endorectal ultrasound according to the ability to completely transverse the tumor with the rigid endorectal ultrasound probe

Tumor completely transversed	Tumor-cell detection only after endorectal ultrasound	
	Yes	No
	10/39 positive (26%)	1/6 positive (17%)*

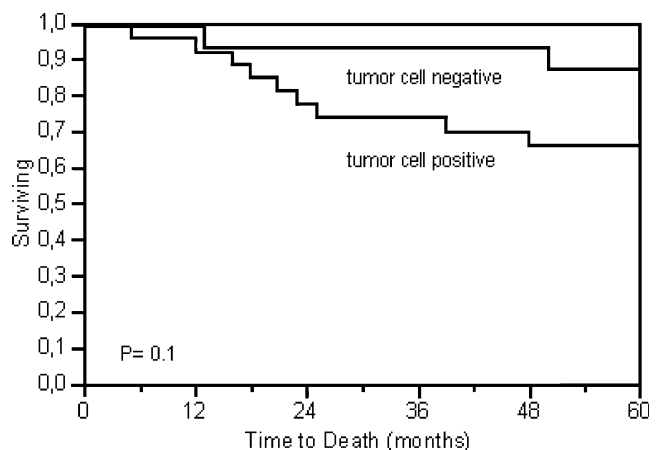
* $p=n.s.$; Fisher's exact test

blood samples taken after endorectal ultrasound was 66.5% compared to 87.5% in patients without tumor cells after endorectal ultrasound (Fig. 1). This difference was not statistically significant ($p=0.1$; log-rank test). The 5-year overall survival rate of patients with tumor cells in blood before endorectal ultrasound was not significantly decreased compared to tumor-cell-negative patients ($p=0.3$; log-rank test) (5-year survival 79.4 vs 67%).

Higher pathohistological tumor stage was significantly correlated to a decreased 5-year survival rate (5-year survival stage I: 100%, stage II: 90%, stage III: 50%, stage IV: 50%, $p=0.001$; Fig. 2).

Discussions

In this study, we investigated the extent of hematogenous tumor-cell dissemination during endorectal ultrasound in patients with primary or recurrent rectal cancer, using a CK 20 RT-PCR assay. In 11 of 45 (24%) patients, tumor cells were detected solely after the endorectal ultrasound examination. The significantly enhanced detection rate after endorectal ultrasound suggests hematogenous tumor-cell spread during or after the procedure, most likely caused by the mechanical manipulation of the rectal tumor with the ultrasound probe. This is in accordance with experimental

**Fig. 1** Influence of tumor-cell detection on overall survival in blood samples taken after endorectal ultrasound ($p=0.1$)

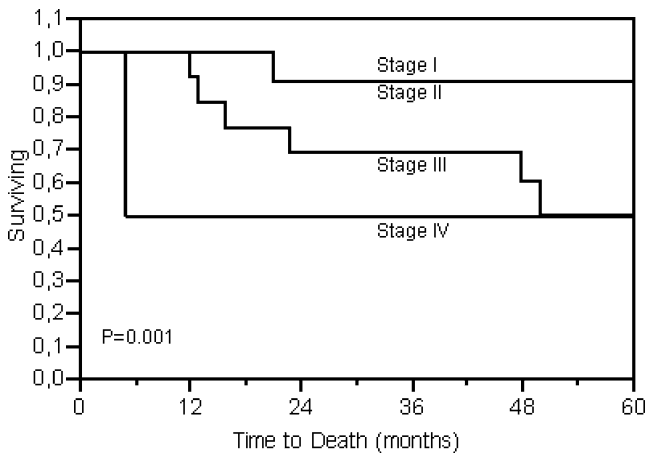


Fig. 2 Influence of UICC stage on overall survival ($p=0.001$)

studies in animal cancer models which have shown that mechanical alteration of malignant tumors leads to a significant tumor-cell shedding into the circulation [27]. Our group recently showed hematogenous tumor-cell dissemination in 44 patients with colorectal cancer undergoing colonoscopy [21]. The comparison of the tumor-cell detection rates after both procedures (24% after endorectal ultrasound vs 14% after colonoscopy) indicates a higher incidence of tumor-cell dissemination after endorectal ultrasound. However, a comparison of the detection rates in these two studies has to be viewed with caution as the data were generated from different, heterogeneous patient populations with regard to tumor localization (only rectal vs all colorectal cancers). Although no other group has yet investigated hematogenous tumor-cell dissemination selectively in patients with rectal cancer, the overall detection rate of disseminated cancer cells in peripheral blood samples (29 of 45 patients, 64%) in our study seems rather high. This may in part also be explained by the particular anatomy of the tumor draining vessels in the rectum compared to those of the more proximal bowel. There are two possible pathways of tumor-cell drainage via blood vessels in rectal cancer: along the iliac vein for tumors of the lower rectum and along the inferior mesenteric and portal vein for tumors of the middle and upper rectum. Our group has already shown that the liver has a potential filtering function for circulating colorectal cancer cells [19]. Therefore, one might hypothesize that patients with low rectal cancers directly draining into the inferior vena cava without blood passage through the liver show a higher rate of disseminated tumor cells in peripheral blood samples. However, statistical analysis of our results did not reveal a difference in median tumor height when comparing tumor-cell-positive and tumor-cell-negative patients.

Although cytokeratin 20 has been demonstrated as a very valuable diagnostic marker for RT-PCR-based protocols [28], there is still a need for further standardization and

quality control among the different protocols for detection of disseminated tumor cells [29]. Therefore, it is essential for every analytic study to demonstrate specificity and sensitivity of the detection system used. The sensitivity of our CK 20 RT-PCR assay was determined in cell spiking experiments; this method allows the detection of ten tumor cells in 10 ml blood [16]. Specificity of our detection system was already adequately demonstrated in previous studies [16, 23]. Moreover, we examined blood samples from 60 healthy volunteers and nine patients with benign gastrointestinal diseases (e.g., rectal adenoma, Crohn's disease, and ulcerative colitis) undergoing endorectal ultrasound; all these consistently tested negative. To allow further interpretation of our tumor-cell detection results, we additionally investigated if hematogenous tumor-cell dissemination may also be caused by enhanced intraluminal pressure in the bowel, e.g., during defecation. This is obviously not the case as an enhanced tumor-cell detection after defecation was not observed.

Because circulating tumor cells in blood of colorectal cancer patients seem to be a frequent phenomenon, the prognostic significance and clinical relevance of hematogenous tumor-cell dissemination have to be further investigated. Several studies already demonstrated a prognostic significance for disseminated colorectal cancer cells in blood [14, 15, 18, 20, 30, 31]. By using a CK 20 RT-PCR, our group showed that there is an enhanced tumor-cell shedding into the circulation during resection of primary colorectal cancer and during resection of colorectal liver metastases [16, 17]. Follow-up of patients with curative resection of colorectal liver metastases revealed that patients with intraoperative hematogenous tumor-cell detection had a significantly poorer outcome [20]. To establish the prognostic and clinical significance of tumor-cell dissemination during endorectal ultrasound, we performed a long-term follow-up of the patients. It is interesting to note that patients with tumor cells in blood samples taken after endorectal ultrasound had a shorter overall survival compared to patients without tumor-cell detection. However, the 5-year overall survival rates between the two groups (66.5 vs 87.5%) were not significantly different but only showed a statistical trend ($p=0.1$). To confirm this trend, a larger patient number needs to be investigated. In addition, a multivariate analysis including all putative prognostic factors has to be performed to assess the true prognostic impact of circulating tumor cells. In our patient cohort, higher pathological tumor stage was significantly correlated to overall survival indicating the validity of our follow-up results.

If our results are substantiated by further studies, alternative staging modalities in rectal cancer should be considered. Although endorectal ultrasound has established itself as a very reliable diagnostic tool for local staging of rectal cancer in the

last decade [32], there is still a debate on the actual accuracy and clinical value of this method in daily practice. Most of the earlier, smaller prospective studies [33–37] demonstrated impressive results for endosonographic staging in rectal cancer, but a recent large analysis of 545 patients [38] only revealed an accuracy of 69% for endorectal ultrasound for assessing the level of wall infiltration (T-classification) in the routine setting. Moreover, magnetic resonance imaging (MRI) is gaining in popularity for preoperative staging of rectal cancer [39]. Brown et al. [40] recently postulated that MRI is superior to endorectal ultrasound in terms of cost and clinical effectiveness. But in the context of tumor-cell dissemination, it will be necessary to investigate whether MRI also causes tumor-cell dissemination by insufflation of water into the rectum.

In conclusion, our results demonstrate that endorectal ultrasound in patients with rectal cancer may lead to enhanced hematogenous tumor-cell dissemination. The prognostic impact of our findings is still unclear and therefore, clinical recommendations for preoperative diagnostic management of rectal cancer cannot be based on the results of this single study.

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