ORIGINAL ARTICLE

Retrospective Study

Circulating cytokeratin-positive cells and tumor budding in colorectal cancer

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Abstract

AIM

To investigate whether circulating cytokeratin-positive (CK+) cells in the mesenteric blood of resected colorectal specimens are prognostic and correlate with tumor budding.

METHODS

Fifty-six colorectal specimens were collected between 9/2007 and 7/2008. Blood from the mesenteric vein was drawn immediately after receiving the fresh and unfixed specimens in the pathology department. After separation of the mononuclear cells by Ficoll-Hypaque

density-gradient centrifugation, cytological smears were immunocytochemically stained for CK18. Tumor budding was evaluated on slides stained for pan-cytokeratin. The identification of $\geq$ 30 buds/1.3 mm^2 was defined as high grade budding.

RESULTS

CK$^+$ cells and clusters were identified in 29 (48%) and 14 (25%) of the samples, respectively. Two cells were identified in one of three non-malignant cases. Clusters were found exclusively in malignant cases. The occurrence of CK$^+$ cells or clusters was not associated with any of the evaluated clinicopathological factors, including surgical technique and tumor budding. Moreover, the occurrence of CK$^+$ cells or clusters had no influence on the cancer-specific survival [75 mo (CI: 61; 88) *vs* 83 mo (CI: 72; 95) and 80 mo (CI: 63; 98) *vs* 79 mo (CI: 69; 89), respectively].

CONCLUSION

CK$^+$ cells and showed neither prognostic significance nor an association with tumor budding. It is very likely that CK18-staining is not specific enough to identify the relevant cells.

Key words: Colorectal cancer; Circulating cells; Tumor budding; Peripheral blood; Survival

Core tip: Blood from the mesenteric vein of 56 colorectal specimens was drawn and evaluated for CK18 positive epithelial cells (CK$^+$). CK$^+$ cells and clusters were identified in a high proportion of cases. However, these cells and clusters were not associated with any of the evaluated clinicopathological factors, including surgical technique and tumor budding. Moreover, the occurrence of CK$^+$ cells or clusters had no influence on the cancer specific survival. Immunocytochemical staining for CK18 does not seem to be a specific marker of mesenteric blood cells for prognostic identification of relevant circulating tumor cells.

INTRODUCTION

Colorectal cancer is a leading cause of cancer-related death, with almost 50000 estimated deaths in the United States in 2016[1]. The prognosis and therapy strongly depend on the UICC tumor stage. Nevertheless, it is well known that a certain proportion of stage Ⅰ/Ⅱ cancers develop an aggressive clinical course. However, approximately 40%

of stage Ⅲ cancers show a favorable outcome despite the occurrence of regional lymph node (LN) metastases[2]. Therefore, alternative or additional prognostic factors are necessary to improve both prognostic estimation and therapeutic stratification in colorectal cancer. The National Comprehensive Cancer Network (NCCN) defined risk factors in stage Ⅱ colorectal cancers that justify the administration of an adjuvant therapy[3]. Several attempts have been made to identify other staging strategies. A very sophisticated approach is the development of multigene assays that could be demonstrated to be prognostic in stage Ⅱ colorectal cancers[4,5]. However, due to the limited evidence concerning their clinical value, these tests were not recommended by the NCCN. The only molecular feature that garnered a recommendation is the microsatellite instability (MSI) status[3]. Very recently, MSI, which is caused by mismatch repair (MMR) deficiency, was demonstrated to be highly predictive for immunotherapy by PD-1 blockade[6,7] Since 2005, Pagès *et al*[8] focused on the host's immune response to the tumor. They developed an immune score based on the densities of CD3$^+$ and CD8$^+$ T-cells and showed that this score is independently prognostic. Currently, a large international multicenter study is ongoing to validate the prognostic role of the immunoscore[9]. A different approach is the detection, quantification and analysis of circulating tumor cells (CTC). These cells circulate in the blood stream or are found in the bone marrow and are believed to be a source of distant metastases. Based on our experiences handling and cannulating fresh colorectal specimens[10,11] for LN isolation, we hypothesized that the detection of epithelial cells in the venous blood of these specimens could be prognostic for the development of hematogenous tumor dissemination and progressive disease. Furthermore, we were interested in whether the occurrence of circulating CK$^+$ cells is associated with tumor budding. Therefore, we collected blood samples from these specimens and evaluated the occurrence of cytokeratin-positive (CK$^+$) cells. In this retrospective study we analyzed the prognostic role of these cells in colorectal cancer.

MATERIALS AND METHODS

Patients

Fifty-six colorectal cancer cases were collected between September 2007 and July 2008. We assumed a strong correlation between the detection of circulating CK$^+$ cells and the occurrence of distant metastases with lethal outcome. An absolute difference concerning lethal outcome of 50% with a power of 0.8 and with Alpha = 0.05 resulted in a calculated sample size of 19 cases in each group (proportions sample size test). Inclusion criteria were proven or suspected cancer, a curative intent and free resection margins. For the survival analysis, only malignant cases with a minimal survival time of 2 mo were included. Follow-up data were provided by the Clinical and Population-Based Cancer Registry of Augsburg. Additional data were acquired from

Figure 1 This image illustrates the blood draw from the mesenteric vein. A standard *i.v.* catheter is used to cannulate the mesenteric vein after removal of the clip.

clinical and laboratory information systems. Informed and written consent was obtained from all patients. The study was approved by the ethics committee of the Landesärztekammer Bayern. The study was performed according to the national rules.

Blood sample collection
Immediately after resection, colorectal specimens were delivered fresh to the in-house laboratory of the Institute of Pathology. The specimens were not opened to avoid contamination by epithelial cells from the mucosa. Manual manipulation was reduced to a minimum to reduce the chance of artificial tumor dissemination. After gentle cleaning, the specimens were placed on a clean board and the main vessels were clamped proximally. Then, the ligation or the clip that was placed by the surgeon was withdrawn. The venous vessel was then cannulated with a standard *i.v.*-catheter (17 Gauge, Braun, Melsungen, Germany). Zero point five milliliter to 8 mL (mean: 3.8 mL; SD: 2.6 mL) of venous blood was drawn using NH_4-heparin blood collection tubes (Sarstedt, Nürnbrecht, Germany) (Figure 1). Then, the blood sample was immediately stored until future use.

Blood sample preparation and immunocytochemistry
The protocol for preparing the cytological samples was initially established for the detection of CK^{+} cells in bone marrow aspirates[12,13]. In brief, the mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation (density, 1.077 g/mole) at $900 \times g$ for 30 min. The cells were then washed and centrifuged at $150 \times g$ for 5 min. Approximately 1×10^6 cells were placed on each glass slide.

To detect epithelial cells within the peripheral blood, a monoclonal antibody against cytokeratin 18 [Clone CK18 (Clone CK2), 1: 100; Chemicon, Hofheim, Germany] was used. The reactions were developed with the alkaline phosphatase anti-alkaline phosphatase technique combined with a new fuchsin stain to indicate antibody binding, as previously described[12,13]. CK^{+} cells and clusters were counted manually (Figure 2). For that all slides were screened by a very experienced technician.
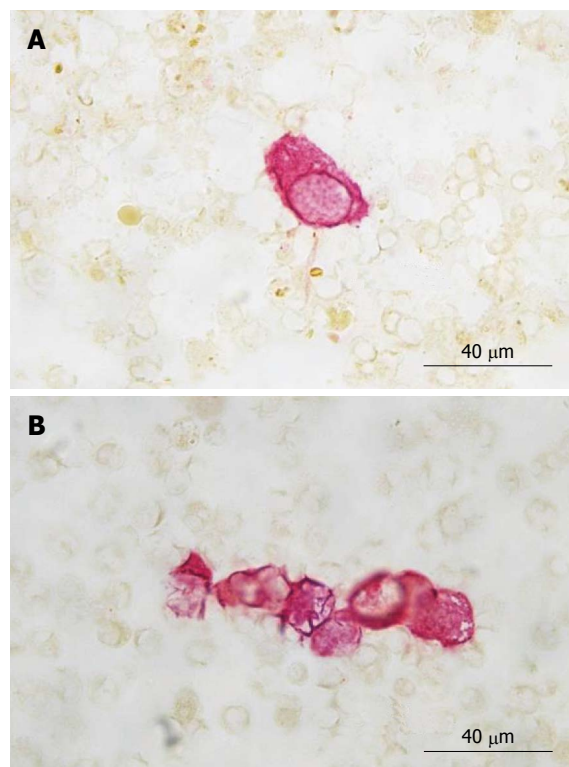

Figure 2 Cytokeratin 18 (Clone cytokeratin 2) cytochemistry. A: A single CK[+] cell is shown in this image; B: A CK[+] cell cluster is shown in this image. CK[+]: Cytokeratin-positive.

All positive cases were confirmed by a hemato-oncologist (DO). Data concerning interobserver agreement between these two investigators are not available.

Histopathological evaluation, immunohistochemistry and tumor budding
Colorectal specimens were macroscopically evaluated after fixing overnight in 10% buffered formalin. LNs were dissected using the methylene-blue method[10,11]; samples from the resection margins, the tumor-region and other conspicuous areas were paraffin-embedded. The slides were stained with hematoxylin and eosin (HE) and evaluated by an experienced pathologist (BM). Based on the HE-morphology, slides were selected for further pan-cytokeratin staining which was performed to enable optimal evaluation of tumor budding. For this evaluation, monoclonal mouse antibody AE1/AE3 was used (dilution 1:50; DAKO). Immunoreactions were developed using a labelled streptavidin-biotin system (DAKO Real detection system). All reactions were performed on a Dako-Autostainer system (DAKO, Glostrup, Denmark).

Tumor budding was evaluated by one pathologist (BM). It was defined as detached single tumor cells or clusters of up to four cells. The cut-off for high-grade budding was adapted from Ueno *et al*[14] and defined as $\geqslant$ 30 buds/20 × magnification (= 1.3 mm²).

Statistical analysis
Metric data were compared using the Mann-Whitney rank sum test. Tabulated data were analyzed with the

Table 1 Clinicopathological data

	Complete collective $n = 56$	CK$^+$ cell negative $n = 27$	CK$^+$ cell positive $n = 29$	*P*-value	CK$^+$ cell cluster negative $n = 42$	CK$^+$ cell cluster positive $n = 14$	*P*-value
Mean age ± SD	70 ± 13	71 ± 11	69 ± 11	0.844	71 ± 12	66 ± 13	0.167
Gender (M:F)	1:1.5	1:1.7	1:1.4	1.0	1:2	1:0.75	0.538
Laparoscopic surgery	15 (27%)	5 (19%)	10 (34%)		10 (24%)	5 (36%)	
Open surgery	41 (73%)	22 (81%)	19 (66%)	0.223	32 (76%)	9 (64%)	0.489
Right colon	21 (38%)	10 (37%)	11 (38%)		16 (38%)	5 (36%)	
Left colon	29 (52%)	13 (48%)	16 (55%)	0.927	21 (50%)	8 (57%)	0.979
Rectum	6 (11%)	4 (15%)	2 (7%)	0.414[1]	5 (12%)	1 (7%)	1.0[a]
Mean LN count ± SD	32 ± 19	29 ± 16	35 ± 21	0.219	30 ± 16	36 ± 25	0.961
LN positivity	20 (36%)	11 (41%)	9 (31%)	0.632	16 (38%)	4 (29%)	0.747
Low grade	33 (59%)	17 (63%)	22 (76%)		28 (67%)	11 (79%)	
High grade	20 (36%)	8 (30%)	6 (21%)	0.576	11 (26%)	3 (21%)	0.735
Non-malignant	3 (5%)	2 (7%)	1 (3%)	n.c.	3 (7%)	0 (0%)	n.c.
pT1/2	16 (29%)	7 (26%)	9 (31%)		11 (26%)	5 (36%)	
pT3/4	37 (66%)	18 (67%)	19 (66%)	0.977	28 (67%)	9 (64%)	0.736
Mean budding ± SD	21 ± 27	20 ± 23	22 ± 30	0.957	19 ± 20	21 ± 26	0.663
High grade budding	16 (29%)	6 (22%)	10 (34%)	0.472	10 (24%)	6 (43%)	0.190
Distant metastases	11 (20%)	5 (19%)	6 (21%)	1.0	8 (19%)	3 (21%)	1.0

[1]Rectum *vs* colon. CK$^+$: Cytokeratin positive; SD: Standard deviation; LN: Lymph node; n.c.: Not calculated.

χ^2 test or Fisher's exact test depending on the expected frequency of the observations. Mean values are given ± 1 standard deviation (SD). Linear regression analysis was performed to calculate correlations between metric data. For the survival analyses, Kaplan-Meier curves were calculated and log-rank tests were performed. ROC analyses were performed to determine the optimized cut-offs. The calculation of the follow-up time was performed according to Schemper and Smith[15]. A *P* value < 0.05 was considered significant. All calculations were performed using the statistics package SigmaPlot 13.0 (Systat, Richmond, VA, United States). The statistical methods of this study were reviewed by Bruno Märkl.

RESULTS

Patients
Fifty-six patients were consecutively collected within 10 mo between 2007 and 2008. The patient characteristics are summarized in Table 1. The mean and median follow-up times were 74 (95%CI: 68; 79 mo) and 80 mo (CI: 77; 83 mo), respectively.

CK$^+$ cells and clusters and their relation to clinicopathological characteristics
CK$^+$ cells were found in 29 (52%) cases with a mean number of 12 ± 14 cells/10^6 cells. One of these cases was non-malignant with two detected CK$^+$ cells. CK$^+$ cell clusters were detected in 14 (25%) cases. The mean number of clusters in positive cases was 3 ± 3 clusters/10^6 cells. No clusters were found in non-malignant cases (Figure 2). There was a strong correlation between CK$^+$ cells and clusters (R = 0.727; *P* < 0.001). Clusters were always accompanied with single CK$^+$ cells.

None of the evaluated clinicopathological features (age, gender, location, LN count, grading, T-stage, metastases) showed an association with the occurrence of

CK$^+$ cells or clusters (Table 1). In particular, neither CK$^+$ cells nor CK$^+$ clusters showed an association with tumor budding (R = 0.180; *P* = 0.185 and R = 0.0637; *P* = 0.647, respectively). The surgical technique (open *vs* laparoscopic technique) did not influence the occurrence of CK+ cells or clusters (Table 1).

Survival analysis
Forty-eight cases met the inclusion criteria for the cancer-specific survival (CSS) analysis. The CSS analysis revealed no significant differences between cases with or without CK$^+$ cells or clusters (Figure 3A and B). Despite the lack of significance, the Kaplan-Meier curve for CK$^+$ cells discriminated between CK$^+$ positive and negative cases with mean CSS times of 75 mo (CI: 61; 88) *vs* 83 mo (CI: 72; 95) (Figure 3A), respectively. The outcome of CK$^+$ cluster positive and negative cases was identical, with mean survival times of 80 mo (CI: 63; 98) *vs* 79 mo (CI: 69; 89) (Figure 3B), respectively. A non-significant trend towards an adverse outcome was found in cases with high-grade tumor budding, with a mean survival time of 71 mo (CI: 53; 89 mo) *vs* 83 mo (CI: 73; 93 mo) (*P* = 0.187, Figure 3C), respectively. ROC analysis identified a certain cut-off that was not positive, *i.e.*, did not reveal a threshold with areas under the curve of 0.51 and 0.55 for CK$^+$ cells and clusters, respectively.

DISCUSSION
In this study, we investigated the prognostic role of circulating CK$^+$ cells and clusters obtained from the mesenteric blood of colorectal specimens. It was our hypothesis that the venous blood from these specimens should be enriched in circulating CK$^+$ positive cells originating from the tumor. We used a technique that was well established for the detection of CK$^+$ cells in the bone marrow of breast, prostate, lung and colorectal cancer
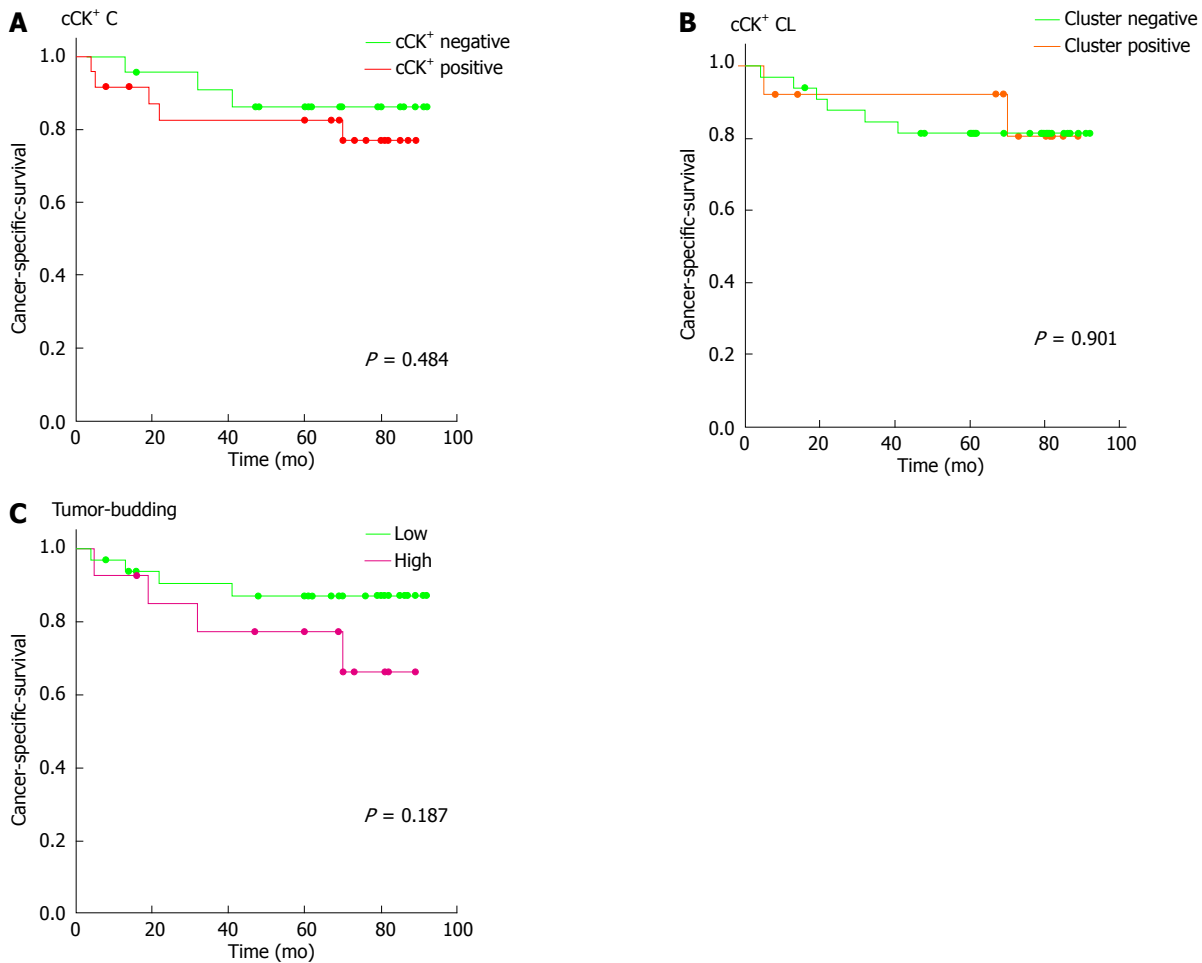
Figure 3 Cancer specific survival. A: Circulating CK⁺ cell negative *vs* positive; B: Circulating CK⁺ cell cluster negative *vs* positive; C: Tumor budding negative/low grade *vs* high grade. CK⁺: Cytokeratin-positive.

patients. Using this method, the detection of cytokeratin-positive cells in the bone marrow could be demonstrated to be prognostic[12,13,16,17].

In this study, we found circulating CK⁺ cells and clusters in the mesenteric blood in a high proportion of cases (52% and 27%, respectively). This positive rate is within the range published in the literature (Table 1). However, it must be noted that only Leather *et al*[18] used immunocytochemistry to detect circulating epithelial cells in the mesenteric blood. In all other identified studies, molecular or flow cytometry techniques were used[19-35]. By using case numbers, we calculated a mean positivity rate in these studies of 43%. When we restricted this calculation to studies that also included stage IV cases, the mean positivity rate was 55%. We detected 2 CK⁺ cells/10⁶ cells in one non-malignant case with diverticulitis. The phenomenon of circulating epithelial cells in the blood in the absence of a malignant tumor has been found by other authors. Pantel *et al*[36] reported the detection of CK⁺ cells in benign colon diseases using two different commercial tests in 11.3% and 18.9% of cases, respectively. In summary, this indicates that the results generated with our immunocytochemical method are comparable to other techniques and are valid.

Despite using an obviously sensitive method, we could not confirm our hypothesis of circulating epithelial cells in the mesenteric blood being prognostic markers of colorectal cancer that correlate with tumor budding. This study is limited by a relatively small case number (*n* = 56) and is therefore underpowered to detect effects that are possibly smaller than expected. We presumed that the prognostic effect of CK⁺ cells was at least as strong as node positivity. Indeed, nodal status revealed a good discrimination with regards to cancer specific survival with a *P* value of 0.058 (data not shown). The strengths of this study are the long follow-up time and the precise evaluation of histological features including an immunohistochemical tumor budding assessment.

Tumor budding is a well investigated prognostic parameter in gastrointestinal cancers. Despite considerable limitations due to the lack of a generally accepted definition and only moderate interobserver agreement, it has been shown in many studies[37,38]. It is believed to be an expression of the epithelial-mesenchymal transition (EMT), which is an important initial step in cancer progression[39]. None of the studies shown in Table 2 investigated the possible relationship between the phenomenon of tumor cell isolation at the invasion front of colorectal cancers and

Table 2 Literature: Circulating tumor cells in the mesenteric blood

Ref.	*n*	Year	Method	Material	Stages	%positive	Prognostic relevance
Leather *et al*[18]	42	1993	ICC	Mesenteric and peripheral blood	I, II, III, IV	15	n.a.
Nakamori *et al*[19]	35	1997	PCR	Mesenteric and peripheral blood	I, II, III, IV	26	uv predictive for recurrence
Luo *et al*[20]	54	1999	PCR	Mesenteric blood	I, II, III, IV	76	Predictive for metastases
Taniguchi *et al*[22]	53	2000	PCR	Mesenteric and peripheral blood	I, II, III	68	uv survival
Yamaguchi *et al*[23]	52	2000	PCR	Mesenteric blood	I, II, III, IV	44	mv survival
Iinuma *et al*[21]	23	2000	MACS	Mesenteric blood	I, II, III, IV	39	uv survival
Fujita *et al*[25]	35	2001	PCR	Mesenteric blood	I, II, III	29	uv recurrence/survival
Etoh *et al*[24]	24	2001	PCR	Mesenteric blood	I, II, III, IV	29	uv recurrence/survival
Guller *et al*[26]	39	2002	PCR	Mesenteric and peripheral blood	I, II, III	$8^1/28^2$	3
Tien *et al*[27]	58	2002	PCR	Mesenteric and peripheral blood	II, III, IV	45^4	n.a.
Akashi *et al*[28]	80	2003	PCR	Mesenteric blood	I, II, III	44	uv metastatic disease; mv no
Nozawa *et al*[29]	41	2003	RTA	Mesenteric and peripheral blood	I, II, III, IV	37	uv predictive for metastatic disease
Sunouchi *et al*[30]	37	2003	PCR	Mesenteric blood	I, II, III, IV	43	uv survival
Zhang *et al*[32]	58	2005	PCR	Bone marrow, portal blood, peripheral blood	I, II, III, IV	74	correlation with stage - no outcome analysis
Sadahiro *et al*[31]	100	2005	PCR	Mesenteric and peripheral blood	I, II, III	$45^5/48^6$	no
Kanellos *et al*[34]	108	2006	PCR	Mesenteric blood	I, II, III	11	uv metastatic disease/survival
Iinuma *et al*[33]	167	2006	PCR	Mesenteric and peripheral blood	I, II, III, IV	$10/34^7$	mv survival
Tseng *et al*[35]	135	2015	FACS	Mesenteric	I, II, III	68	mv survival

[1]Blood; [2]Blood and peritoneal fluid; [3]No separate evaluation for blood samples; [4]Multiple measurements; [5]Mesenteric blood; [6]Peripheral blood; [7]Mesenteric. n.a.: Not available; uv: Uni-variable; mv: Multi-variable; ICC: Immunocytochemistry; PCR: Polymerase chain reaction; MACS: Magnetic activated cell sorting; FACS: Fluorescence activated cell sorting.

the occurrence of CTCs in the blood. Moreover, a literature search within the Medline, Embase and Google Scholar databases did not reveal an investigation that addressed this topic. Cao *et al*[40] postulated in a review that EMT leads to tumor budding and subsequent blood vessel invasion. However, this is not supported by other references. To us, it seemed quite obvious that a correlation between these two factors exists. However, we were not able to confirm this hypothesis. We could not identify a correlation between tumor budding and circulating CK$^+$ cells and could not confirm that a combination of tumor budding and CK$^+$ cells was prognostic. Tumor budding alone discriminated clearly between two prognostic groups (Figure 3C). However, significance was likely not achieved due to the small sample number.

The data concerning the prognostic significance of CTCs and disseminated tumor cells (DTCs) are conflicting[41]. However, there is growing evidence that CTC/DTCs are of prognostic significance. Two commercial tests based on immunomagnetic separation targeting EpCAM (BerEp4) are currently available. They have proven to be prognostic, particularly in the metastatic stage of different cancers including colorectal cancer[42,43]. Two meta-analyses addressed this topic. Katsuno *et al*[44] restricted their analysis to molecularly detected CTCs in mesenteric blood and included 9 studies. They found a favorable outcome in patients negative for CTCs [hazard ratio (HR) 0.4-0.08][44]. Rahbari *et al*[45] included 36 studies with a total 3094 patients. They also identified a prognostic effect of CTCs. However, stratification according to the sampling compartment revealed that CTCs of peripheral blood were prognostic but those of the mesenteric bone marrow blood were not[45]. Similarly, our study found that the identification of CK$^+$ cells or clusters had no prognostic

effect. In addition, the approach using ROC analyses to identify a certain cut-off of cells which might be prognostic failed.

CTCs seem to comprise different cell types of neo-plastic and non-neoplastic origin. Moreover, it is very likely that cells derived from cancer have different potential to escape from immunogenic destruction and to establish tumor growth at a distant site. Depending on the compartment, cells may undergo a change in their phenotype[40,41,46]. As mentioned before, EMT is a hallmark process in cancer progression and is associated with impaired outcome[46,47]. Cells undergoing EMT lose their epithelial phenotype and gain mesenchymal features. The use of methods optimized for the detection of epithelial cells is prone to fail in the detection of all CTCs. Moreover, these methods may fail to detect the most relevant cells[48]. Currently, the most interesting cells in this context are cells with stem-cell features. The realization of a fast, exact and cost effective technical method to detect these cells is likely the most promising approach.

In this study, we hypothesized that the immuno-cytochemical detection of CK$^+$ cell in the mesenteric blood of colorectal cancer specimens correlates with tumor budding and could serve as an easy to determine prognostic factor. Drawing the blood after resection would avoid delay and additional risk during the operation. None of these hypotheses could be confirmed in our study. Given the current literature, peripheral blood and not mesenteric blood is the optimal material for the detection of CTCs. More sophisticated techniques including molecular approaches are relatively expensive and their availability is limited. Nevertheless, they have the potential to detect exactly the cells which are most likely to be relevant to the clinical

course of the disease. Immunocytochemical detection seems to be less specific and is not favorable.

ACKNOWLEDGMENTS

COMMENTS

Background
Colorectal cancer is one of the most common cancers in men and women. Its prognosis depends mainly on the (UICC-) tumor stage. However, it is also known that certain proportion of cancers with otherwise favorable features and low stages show an aggressive clinical course while locally advanced cancers so not relapse. It is accepted that the detection of circulating tumor cells has the potential to improve the prognosis estimation not only in colorectal cancer.

Research frontiers
The main topic in the research field of circulating tumor cells is the influence of the different compartments (peripheral blood, mesenteric blood or bone marrow) on the clinical significance of the detected cells. Other important questions are the methods for the assessment and the type of cells (*e.g.*, stem cells) which are most informative to predict the outcome.

Innovations and breakthroughs
The innovation of this study is the evaluation of the blood draw from resected specimens. A direct correlation with tumor budding as a source for the circulating tumor cells is also a new approach.

Applications
Because the authors' hypotheses could not be confirmed, the main conclusions are that mesenteric blood is probably not the best compartment for the identification of the relevant cells and more sophisticated methods may be superior over immunocytochemistry. Molecular techniques are more sensitive in detecting cells with a high potential to serve as the origin for distant metastases.

Terminology
Circulating tumor cells are cells that lost its cohesion to the primary tumor mass and achieved access to the vascular system including the bone marrow.

Peer-review
This is an interesting manuscript which appears to add to the existing body of literature around this subject. The design is clear.

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