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Prognostic relevance of disseminated tumour cells in bone marrow of patients with transitional cell carcinoma

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ABSTRACT

Objective: This prospective study is the first immunocytochemical investigation of the frequency and prognostic value of CK+ tumour cells in the bone marrow of patients with transitional cell carcinoma (TCC).

Methods: Bone marrow aspirates from 228 TCC patients were taken preoperatively. Cytospins were made and stained by immunocytochemistry using the monoclonal antibodies CK2 and A45-B/B3. 27 patients with no evidence of any malignant disease served as control group.

Results: CK+ tumour cells were detected in 28% (63/228) of the TCC patients. No CK+ cells (0/27) were detected in the control group. In multivariate analysis the detection of $\geqslant 3$ CK+ cells in bone marrow was an independent prognostic factor (hazard ratio = 2.7, $p < 0.05$) in patients with T2–4 tumour classification.

Conclusion: Disseminated CK+ cells play a role in the biology of tumour spread of TCC, and their immunocytochemical detection can be useful in assessing the prognosis of TCC patients with an invasive tumour.

1. Introduction

Several investigations in patients with different tumours (e.g. colorectal cancer, mammary carcinoma, head and neck cancer) have demonstrated the prognostic value of disseminated epithelial tumour cells in bone marrow detected by immunocytochemistry using anti-cytokeratin antibodies.[1–4] To our knowledge, the prognostic relevance of these disseminated cytokeratin-positive (CK+) cells has never been analysed in transitional cell carcinoma (TCC). The majority of tumours are localised in the urinary bladder. The estimated number of incident bladder cancer cases in the European Union was 116,100 in the year 2004.[5] Typical features of TCC are multiple localisations and frequent tumour recurrence, often accompanied by tumour progression from a superficial to an invasive tumour stage. In the current prospective study, we analysed the prognostic potential of CK+ cells in bone marrow aspirates from 228 TCC patients to elucidate the changes in tumour cell shedding that might occur dependent on tumour stage and grade and that could give additional information about the patients' individual outcome.

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2. Patients and methods

2.1. Patients and bone marrow aspirates

Between June 1990 and July 2001 bone marrow aspirates were taken from 279 unselected patients with transitional cell carcinoma (TCC) after written informed consent was obtained from every patient. The study was approved by the local ethics committee. A total bone marrow volume from 2 to 12 ml was taken from both anterior iliac crests immediately before the start of the surgical procedure (transurethral resection, laser coagulation, nephroureterectomy or cystectomy). Bone marrow analysis and follow-up examinations were performed by different individuals. Patients were re-examined for follow-up either as outpatients at the Department of Urology, Klinikum Großhadern, or by local urologists. Tumour-associated death was the relevant event for prognostic analysis. Additionally, the time interval between bone marrow aspiration and the next tumour recurrence or tumour progression was used for outcome analysis. 38 patients were excluded from further analysis because a second tumour (prostate carcinoma, colon cancer and others) was diagnosed during the follow-up period. A second tumour could have been the source of disseminated tumour cells causing a false-positive result. A positive staining reaction of the isotype control occurred in 5% (13/241) of the remaining patients. They were excluded from the study, leaving 228 cases for further analysis (213 patients with bladder TCC and 15 patients with renal pelvis TCC; age ranged from 19 to 91 years, median age 63 years, male-to-female ratio 3.3:1). In general, patients with superficial tumours (Ta, T1) received a single instillation therapy with Epirubicin or Mitomycin C after transurethral resection (TUR). In high-risk cases (e.g. 15 patients with T1G3 tumour), a second TUR was performed, followed by instillation therapy with BCG (Bacille Calmette-Guerin) for several months. In patients with invasive tumour (T2–4), early cystectomy (within a few days after diagnosis) was the standard procedure. In some cases, mostly due to a decreased performance status and/or high co-morbidity, a primary radiotherapy was performed instead. Palliative cystectomy was performed in few cases to manage severe haematuria. The majority of patients with advanced disease received a cisplatin-based systemic chemotherapy (MVAC = methotrexate, vinblastine, doxorubicin, cisplatin; MVEC = methotrexate, vinblastine, epidoxorubicin, cisplatin), some patients received gemcitabine or gemcitabine/cisplatin. There was no change of therapeutic strategy during this study.

2.2. Bone marrow preparation and immunocytochemistry

Per aspirate 4×10^6 mononuclear cells were used for the immunocytochemical analysis of cytokeratin positive (CK+) cells. Erythrocytes in the aspirate were removed by ammonium chloride lysis buffer. Then, density gradient centrifugation in Ficoll–Paque (Seromed, Berlin, Germany) was performed and the mononuclear cells were obtained from the interphase. 1×10^6 cells per spot were centrifuged on a glass slide, air-dried overnight and stored at −80 °C. 2×10^6 cells per bone marrow sample were used for the specific immunocytochemical analysis with monoclonal antibodies, the remaining 2×10^6 cells from every sample were used for the isotype-specific negative control staining with an irrelevant mouse IgG1 antibody (MOPC 21, Sigma, St. Louis, MO, USA). Nine years after our study started, this number of cells was recommended as standard by the ISHAGE Working Group for Standardisation of Tumour Cell Detection that was founded to standardise and optimise procedures used for the detection of minimal residual disease.[6] For detection of CK+ cells the monoclonal antibodies CK2 (Boehringer Mannheim, Mannheim, Germany) and A45-B/B3 (Micromet, Munich, Germany) were used. CK2 is directed against cytokeratin 18, which is expressed in a wide variety of epithelial cells and carcinomas, while A45-B/B3 recognises a common cytokeratin epitope in various cytokeratins, such as cytokeratin 8, 18 and 19. Cytospins from the colon carcinoma cell line HT-29 with constitutive cytokeratin expression were used as positive control in each immunocytochemical staining procedure. Staining of all cytospins was performed according to the alkaline phosphatase-anti-alkaline phosphatase (APAAP) protocol described by Cordell and colleagues.[7] After incubation with the primary antibody the cytospin slides were washed and incubated with rabbit-anti-mouse immunoglobulin polyclonal antibodies (Dako, Hamburg, Germany). These antibodies were labelled with preformed APAAP complexes. After the addition of chromogen (naphthol-AS-biphosphate and new fuchsin) the CK+ cells stained red (Fig. 1). In addition to the positive immunocytochemical staining result every positive cell was evaluated according to morphologic criteria. Only cells with positive staining and morphologic criteria of malignancy such as enlarged or atyp-

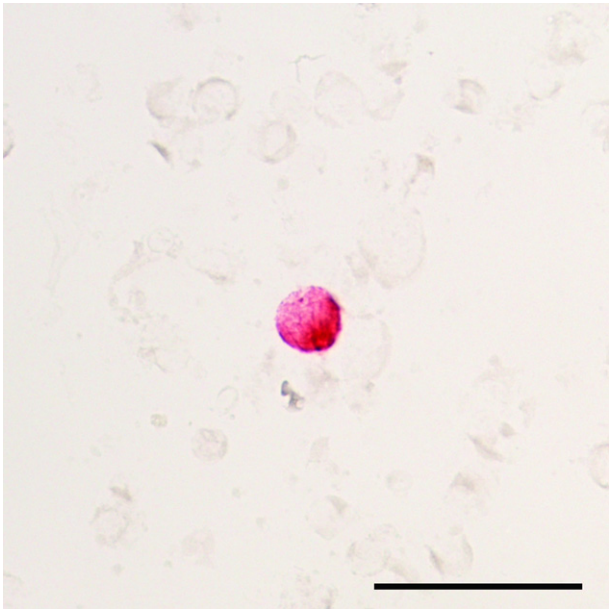

Fig. 1 – Cytokeratin-positive (CK+) cell in bone marrow aspirate of a patient with transitional cell carcinoma. Epithelial tumour cells showed red staining after immunocytochemistry, whereas haematopoietic bone marrow cells remained unstained. Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ical nucleus or cluster formation were assessed as tumour cells. This procedure is consistent with the aforementioned ISHAGE recommendations.[6] All slides were examined by two independent reviewers (with several years of experience in assessment of immunocytochemically stained bone marrow preparations from other studies and cooperation in this field) who were blinded to the results of the other one. Differing results in some slides were discussed extensively, and agreement could be achieved in every case.

2.3. *Statistical analysis*

To achieve 90% power for the detection of a 1.5 hazard ratio in patients with cytokeratin-positive bone marrow status (α level = 0.05), it was calculated by power analysis that an enrolment of at least 148 evaluable patients was required. We intended to expand patient recruitment and follow-up time above the calculated values for optimal study power. The χ^2 test was used to examine the association between cytokeratin status (positive versus negative) and categorised parameters such as tumour classification and grade (TNMG) classification or gender. The influence of cytokeratin status and other factors on prognosis (tumour-specific death as relevant event) was determined by Kaplan–Meier analysis and log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. For all tests $p < 0.05$ was regarded as significant. All statistical calculations were performed using the software STATISTICA for Windows (release 7.1, StatSoft, Tulsa, OK, USA).

3. Results

Cytokeratin-positive cells were detected in 28% (63/228) of the bone marrow samples from TCC patients. The majority of CK+ cases (71%, 45/63) showed only one or two cytokeratin-positive cells in the bone marrow sample (Fig. 2). The range of CK+ cells in the positive cases was 1–100. In January 1994 the antibody CK2 (directed against cytokeratin 18) was replaced by the antibody A45-B/B3, which recognises several cytokeratin subtypes. The expected increase in sensitivity could not be confirmed since there was no significant difference in the proportion of CK+ cases [26% (32/121) and 29% (31/107), respectively; $p = 0.67$] and in the distribution of CK+ cells per positive sample between the CK2 and the A45-B/B3 group, and there was no significant difference in clinical outcome. The proportion of CK+ cases in relation to patients' characteristics is shown in Table 1. No significant difference for the proportion of CK+ versus CK negative cases could be detected regarding gender and age. There were more CK+ cases amongst patients with a positive lymph node status [N0: 26% (52/200) and N+: 39% (11/28), $p = 0.14$], and the proportion of CK+ cases increased with the T classification ($p = 0.05$). In patients with metastatic disease (M1) CK+ bone marrow aspirates were detected significantly more often than in M0 patients ($p = 0.03$). In addition, patients with a G3 tumour presented significantly more often with a positive bone marrow status than patients with a lower tumour grade ($p = 0.02$).

The median follow-up time was 63 months. The time interval between tumour resection and the next tumour recurrence was longer in patients with a negative bone marrow status than in CK+ patients (12.0 months and 7.0 months, respectively; $p = 0.15$, Fig. 3). For all other follow-up calculations, an event was defined as a tumour-associated death. In 23% (52/228) of the patients there was an event. There was no significant difference between CK+ cases and patients with a negative bone marrow result [27% (17/63) and 21% (35/165), respectively; $p = 0.35$] regarding tumour related death. The log-rank test showed significant influence of local tumour extent, lymph node status, distant metastases and tumour grade on the clinical outcome within the TCC patients (T: $p < 0.001$, N: $p < 0.001$, M: $p < 0.05$, G: $p < 0.01$). When discriminating between CK+ patients and patients with a negative bone marrow immunocytochemistry result, there was no difference in prognosis (log-rank $p = 0.26$, Fig. 4a). A similar result was obtained when analysing only the patients with T2–4 tumours (log-rank $p = 0.27$, Fig. 4b). Comparison of

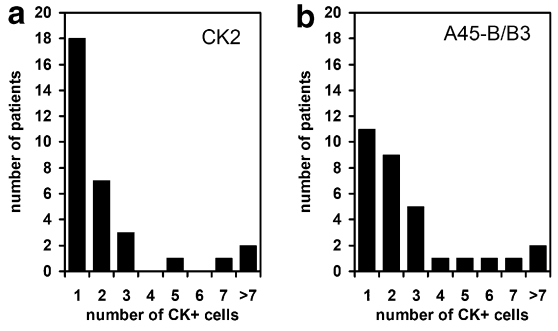

Fig. 2 – Distribution of the number of CK+ cells per case in the group of 63 CK+ TCC patients. In the majority of cases, only one or two CK+ cells were detectable, without significant difference between both antibodies (a: CK2, b: A45-B/B3).

Table 1 – Distribution of cytokeratin immunocytochemistry results in relation to patients' characteristics

	% CK+ (number/total number)	p (χ^2 test)
Gender		
Male	26 (46/175)	0.409
Female	32 (17/53)	
Age		
⩾62.9 years (median)	25 (28/114)	0.300
<62.9 years	31 (35/114)	
Tumour classification		
Ta/is	18 (17/93)	0.051
T1	30 (18/60)	
T2	38 (15/40)	
T3–4	37 (13/35)	
N0	26 (52/200)	0.141
N+	39 (11/28)	
M0	27 (60/224)	0.033
M1	75 (3/4)	
G1–2	23 (33/146)	0.023
G3	37 (30/82)	

TNMG: tumour classification (8) and grade, CK+ = cytokeratin-positive cells in bone marrow detected.

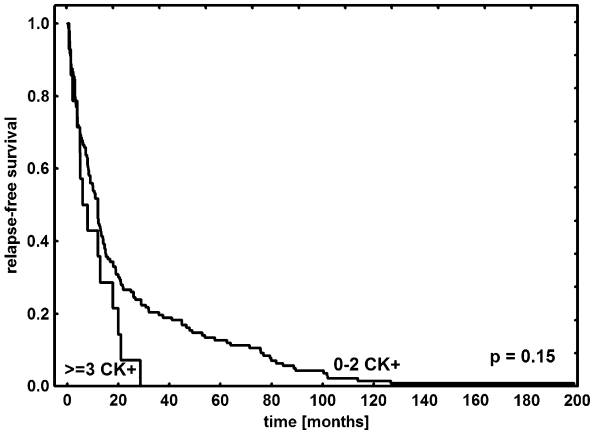

Fig. 3 – Relapse-free survival of TCC patients with a threshold of three or more cytokeratin-positive (CK+) cells in bone marrow sample. There is a trend indicating a longer relapse-free interval for patients with less than three CK+ cells (log-rank test: $p = 0.15$).

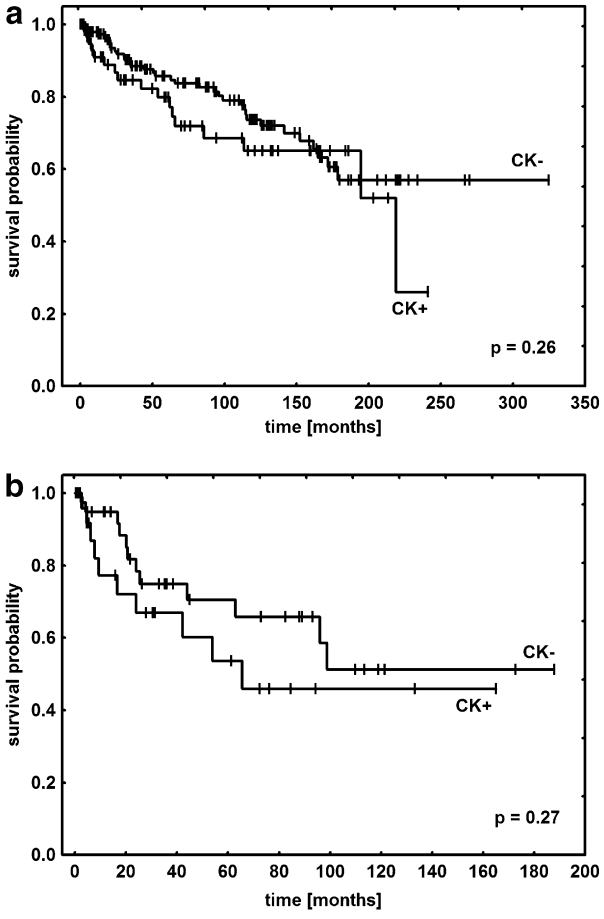

Fig. 4 – Survival analysis of TCC patients. (a) All patients ($n = 228$): log-rank test showed no difference between patients with cytokeratin-positive (CK+) and cytokeratin-negative (CK–) bone marrow status. (b) Patients with T2–4 classification ($n = 75$): no significant difference between CK+ and CK– patients.

patients with at least 3 CK+ cells in their bone marrow sample (CK3+) to the other patients revealed a trend indicating a worse clinical outcome for the CK3+ patients, but the difference was not significant (log-rank $p = 0.11$, Fig. 5a). This threshold was then applied to the patients with a TCC that exceeds the basal membrane (T1–4, $n = 135$). In these patients, there was good outcome discrimination between the CK3+

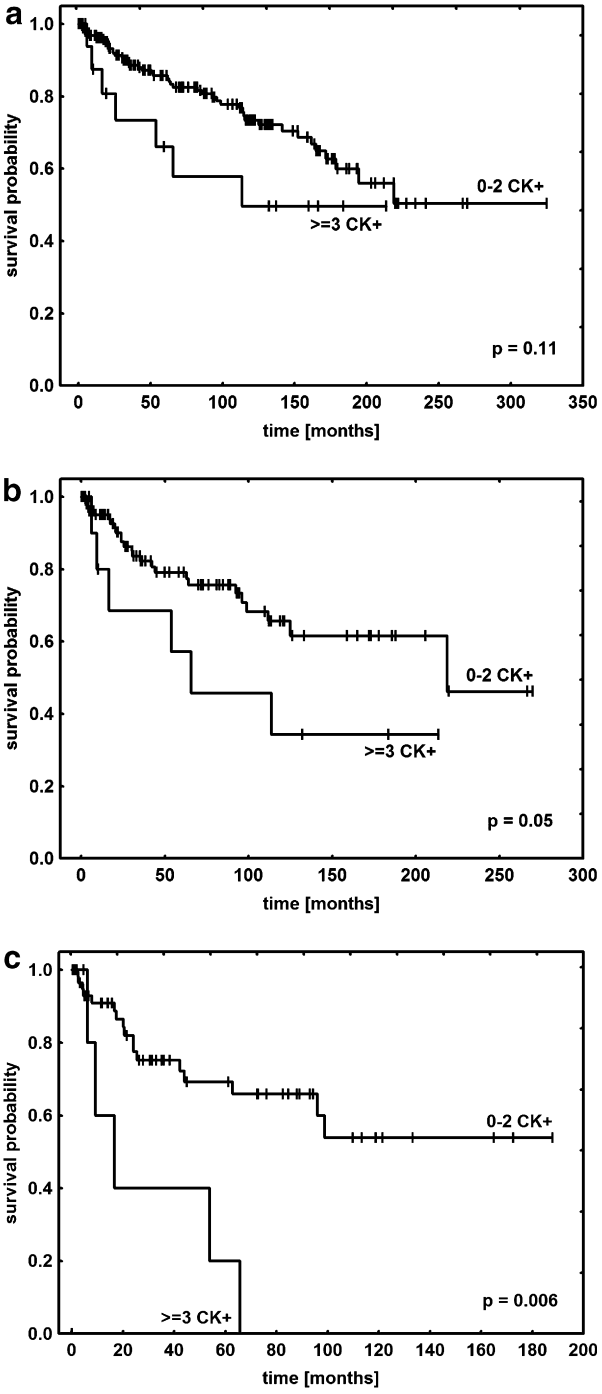

Fig. 5 – Survival analysis of several TCC subgroups using three or more CK+ cells per bone marrow sample as threshold. (a) All patients ($n = 228$): log-rank test: $p = 0.11$; (b) patients with T1–4 classification ($n = 135$): $p = 0.05$ and (c) patients with T2–4 classification ($n = 75$): $p = 0.006$.

Table 2 – Multivariate analysis (Cox regression model) of TCC patients with T2–4 classification

	Hazard ratio	95% confidence interval	*p*
T	0.6	0.3–1.4	0.259
N	2.5	1.2–5.3	0.017
M	2.3	0.2–29.9	0.519
G	1.6	0.6–4.3	0.363
mAb	0.8	0.3–1.9	0.579
CK3+	3.5	1.0–11.9	0.042
N	1.7	0.9–2.9	0.089
CK3+	2.7	1.0–7.4	0.044

TNMG: tumour classification (8) and grade, mAb: the monoclonal antibody used in immunocytochemistry, CK3+: detection of at least three cytokeratin-positive cells in the bone marrow. CK3+ remains significant when limiting the analysis on N status and CK3+, which are significant factors in the first regression model.

group and those patients with 0–2 CK+ cells in their bone marrow aspirate (log-rank $p = 0.05$, Fig. 5b). The next analysis included only patients with T2–4 tumours ($n = 75$); amongst these patients the CK3+ group ($n = 7$) had a significantly worse prognosis than the patients with 0–2 CK+ cells in their bone marrow sample ($n = 68$; log-rank test = 0.006; Fig. 5c). All patients with a minimum of 3 CK+ cells died within 66 months. The median survival time in this group was 17 months, whereas in the patients with 0–2 CK+ cells the median survival was not reached during the follow-up period. The influence of this CK+ detection threshold was tested by multivariate analysis (Cox proportional hazards regression model with TNM classification,[8] grade and antibody, Table 2). In this Cox regression model, only N status and CK3+ were significant factors. In a second model that included only N status and CK3+, the CK3+ bone marrow status remained significant. Therefore, the detection of at least three CK+ cells in bone marrow is an independent prognostic factor with a hazard ratio of 2.7 (95% confidence interval 1.0–7.4, $p < 0.05$).

4. Discussion

Patients with TCC show a wide variability in the course of their disease, even when they have comparable tumour stage and grade. In the past years, many studies have examined the detection and prognostic relevance of disseminated tumour cells in patients with various tumours. Most of the monoclonal antibodies used in these studies were directed against cytokeratin to detect epithelial cells in mesenchymal tissue such as bone marrow or peripheral blood. In this prospective study, the frequency and clinical relevance of disseminated CK+ cells in patients with TCC was analysed for the first time. Bone marrow samples were chosen for analysis because there is evidence for a higher frequency of disseminated tumour cells in bone marrow as compared to venous blood.[9]

Initially the monoclonal antibody CK2 was used, which is directed against cytokeratin 18. To increase the sensitivity of tumour cell detection, CK2 was replaced in January 1994 by A45-B/B3, which recognises a common epitope on various cytokeratins, including cytokeratin 8, 18 and 19. The proportion of CK+ cells was 3% higher in the A45-B/B3 group than in the CK2 group, but this difference was not significant (29% versus 26%, $p = 0.67$). A possible explanation for the small increase in sensitivity might be the down regulation of cytokeratin 18, which has been demonstrated for different carcinomas but not for TCC so far.[9,10]

In our cohort of TCC patients, the CK2 and the A45-B/B3 group showed no significant difference in clinical outcome. This finding is in contrast to results in patients with breast cancer and prostate cancer, in whom A45-B/B3 demonstrated to have a significantly higher sensitivity than CK2.[1,11]

There is evidence for the malignant origin of epithelial cells in bone marrow in several studies. False positive staining of haematopoietic cells could be excluded by double staining immunocytochemistry with CK2 antibody and a monoclonal antibody directed against CD45.[2] To achieve optimum specificity in screening of bone marrow for disseminated tumour cells, morphological criteria related to the malignant origin of CK+ cells (e.g. enlarged or atypical nucleus, cell cluster) have to be considered in addition to the staining result.[6] Co-localisation of cytokeratin and prostate specific antigen (PSA) in bone marrow aspirates from prostate cancer patients was demonstrated by Riesenberg and colleagues.[12]

Pantel and colleagues could show that detection of CK+ cells in bone marrow aspirates from patients without a carcinoma is a very rare event.[13] By comparison with the latter study no patient (0/27) from a control group without malignant disease showed a CK+ bone marrow aspirate according to our previous paper on the immunocytochemical detection of disseminated tumour cells in patients with TCC.[14]

The low frequency of CK+ cells in mesenchymal tissue samples of cancer patients makes the detection of these cells difficult. Standard immunocytochemistry methods allow detection of one tumour cell in 10^6 bone marrow cells.[15] In our study, 71% of the 63 positive patients had only one or two detectable tumour cells (Fig. 2). New experimental approaches, such as magnetic bead enrichment, could increase detection frequency of CK+ cells and facilitate their further characterisation.[16,17] Furthermore, novel automated enrichment methods now allow high-throughput analysis on peripheral blood samples from tumour patients, thus making the conduction of large prospective studies more feasible.[18,19]

In the present study, CK+ cells in bone marrow aspirates from TCC patients were detectable in 28% of the cases. The clinical outcome of patients with CK-positive and CK-negative bone marrow samples was not significantly different, although there was a trend towards a better outcome for patients without CK+ cells (Fig. 3). When applying a higher threshold (detection of at least three CK+ cells; Fig. 4), the difference in clinical outcome between the groups became more evident ($p = 0.11$). The discrimination between the different prognostic groups increased when analysing tumours that invaded beyond the basal membrane ($pT \geqslant 1$: $p = 0.05$) and was significant in patients with tumour classification $T \geqslant 2$ ($p = 0.006$). This was confirmed by multivariate analysis. The detection of at least three CK+ cells in T2–4 TCC patients had a hazard ratio of 2.7 in the Cox regression model ($p < 0.05$) and was independent from TNM classification or

grade and from the antibody used for immunocytochemistry. These results are consistent with a previous study on metastatic renal cell carcinoma (RCC) patients where the same threshold of CK+ cells in bone marrow was an independent prognostic factor.[20] Obviously, a certain minimum concentration of disseminated tumour cells in the bone marrow (which may vary in relation to the detection method) is a surrogate marker for the malignant potential of an already systemic tumour disease, independently of tumour staging and differentiation. Only in TCC patients with T2–4 classification did the detection of at least 3 CK+ cells have a significant prognostic impact. This finding may be explained firstly by the fact that metastatic disease in TCC patients is nearly exclusively seen in muscle invasive or locally advanced disease as blood supply in the muscle layers of the bladder seems to be crucial for metastatic tumour spread. This is reflected by the association between T classification and the frequency of CK+ results. Secondly, the elimination of a certain number of disseminated tumour cells by immunologic response mechanisms may serve as a possible explanation for the finding that numerous disseminated tumour cells are necessary to create a relevant number of potential seeds for tumour relapse, progression and poor outcome.

The prognostic relevance of disseminated tumour cells has been examined in a variety of tumour types with heterogeneous results. In a meta-analysis performed on 20 studies on the prognostic influence of CK+ cells in bone marrow aspirates of overall 2494 patients with different carcinomas, the detection of these cells was a predictor for relapse-free survival in 14 of 20 studies using univariate analysis and in 5 of 11 studies using multivariate analysis.[21] The published proportion of tumour patients with a positive bone marrow status ranges from 20% to 50%.[22] Our data indicate that the sole detection of cytokeratin-positive tumour cells in bone marrow aspirates has no significant influence on prognosis. However, the difference with regard to survival probability becomes significant in univariate and multivariate analysis, when a higher threshold (at least three CK+ cells) is applied. Our findings are confirmed by the results of a prospective study about the prognostic relevance of circulating tumour cells in the peripheral blood of breast cancer patients; in that study, the detection of at least five tumour cells per blood sample was an independent prognostic parameter.[23] Our data can help to identify TCC patients with a high risk for tumour progression and may assist in developing a risk-adapted therapy. One can speculate that the concentration of CK+ cells in peripheral compartments reflects the malignant potential of the individual disease. It is still unknown if all these cells are of equal importance for the clinical outcome or if there is a specific subpopulation of disseminated tumour cells that initiates further progression, the so-called cancer stem cells. The technology to analyse the transcriptome and genome of single cells has recently been established.[24] There is evidence for the hypothesis that disseminated tumour cells with certain characteristics are crucial for the patients' outcome.[25,26] Once such characteristics are revealed, disseminated tumour cells can be specifically targeted in a minimal-residual disease situation by novel therapy strategies such as antibody therapy. Assuming that these disseminated tumour cells might represent the target for an effective postoperative adjuvant systemic therapy,[27] the patients for this therapy could be selected according to their risk determined by the presence of CK+ cells in peripheral compartments.

In summary, the results of this first prospective immunocytochemical study addressing the prognostic value of disseminated cytokeratin-positive (CK+) tumour cells in bone marrow of TCC patients indicate that these cells play a role in the biology of tumour spread of TCC, especially in invasive tumours. The immunocytochemical detection of CK+ cells can be useful in the assessment of clinical outcome in TCC patients with advanced tumour stage, and it can optimise patients' stratification for a risk-adapted therapy. Further characterisation of these CK+ cells is necessary to evaluate their individual malignant potential and to use them as potential therapeutic targets for a novel systemic therapy.

Conflict of interest statement

None declared.

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