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European Association of Urology

Platinum Priority – Bladder Cancer

Long-term Follow-up of Bladder Cancer Patients with Disseminated Tumour Cells in Bone Marrow

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Article info

Article history:
Accepted December 10, 2010
Published online ahead of print on December 22, 2010

Keywords:
Disseminated tumour cells
Bone marrow
Bladder cancer
Metastasis

Abstract

Background: The clinical relevance of polymerase chain reaction (PCR)–based techniques for detection of disseminated tumour cells (DTCs) in the bone marrow of bladder cancer (BCa) patients is still under debate, as data on long-term follow-up analysis have not yet been published.

Objective: The aim of the present prospective study was to assess the prognostic significance of DTCs detected by cytokeratin-20 (CK20) reverse-transcriptase PCR in bone marrow from BCa patients undergoing radical cystectomy (RC).

Design, setting, and participants: Bone marrow samples from 51 BCa patients with high-risk non–muscle-invasive or muscle-invasive urothelial carcinoma were drawn from the anterior iliac crest prior to RC. CK20-positive cells in bone marrow were detected by qualitative RT-PCR.

Measurements: BCa patients with CK20 status were analysed with respect to the end points tumour progression and cancer death. A multivariate Cox regression analysis was performed to determine independent prognostic factors for progression-free survival (PFS), tumour-specific survival (TSS), and overall survival (OS).

Results and limitations: CK20-positive cells were detected in 16 of 51 (31.4%) BCa patients of all stages. BCa patients with CK20-negative status displayed a 7-yr PFS rate of 64% versus 35.2% for CK20-positive patients ($p = 0.007$). TSS was significantly shorter in the CK20-positive group, with a 7-yr survival rate of 46.9% compared to CK20-negative patients with 70.2% ($p = 0.012$). The 7-yr OS rate of 37.5% for CK20-positive patients was significantly $<$65.7% in the CK20-negative group ($p = 0.006$). A subgroup analysis of lymph node–negative patients (pN0) discriminated by CK20 status revealed significant differences in PFS, TSS, and OS. In a multivariate analysis, CK20-status provides independent prognostic information with respect to all three survival end points.

Conclusions: BCa patients with positive CK20 status in bone marrow represent a high-risk subgroup reflected by an unfavourable outcome in the long-term analysis.

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0302-2838/$ – see back matter © 2010 Published by Elsevier B.V. on behalf of European Association of Urology. doi:10.1016/j.eururo.2010.12.014

1. Introduction

The prognosis of bladder cancer (BCa) patients is predominantly determined by the occurrence of distant metastasis. Despite intentionally curative radical cystectomy (RC), the 10-yr recurrence-free survival rate for patients with organ-confined lymph node–negative BCa ranges between 70% and 82% [1,2]. Metastatic relapse is probably caused by occult micrometastasis in distant organs at the time of primary diagnosis, which is not detectable with high-resolution imaging techniques. With the availability of more sensitive molecular diagnostic methods such as immunocytochemistry and reverse-transcriptase polymerase chain reaction (RT-PCR), bone marrow has been identified as a common homing site for disseminated tumour cells (DTCs) derived from various types of epithelial tumours [3]. In breast cancer, a meta-analysis consisting of >4700 patients demonstrated that the detection of DTCs in bone marrow at the time of initial diagnosis is a significant and independent prognostic factor with respect to tumour-specific survival (TSS) and overall survival (OS) [4].

During the past decade, >15 publications have investigated bladder tumour cells in peripheral blood and bone marrow using RT-PCR–based strategies [5]. Uroplakins, mucins, and cytokeratins were chosen as putative BCa cell markers [6]. Initial results have shown an association between histopathologic tumour stage and the presence of DTCs at the time of diagnosis. However, a long-term follow-up for disease outcome is still missing to evaluate the prognostic significance of the molecular marker and the value of the method. In our first study in 2001, we presented an approach for detecting DTCs in bone marrow from BCa patients using cytokeratin-20 (CK20) as a molecular marker [7]. We have developed a standard operating protocol for sample collection and a preparation procedure as well as optimising the sensitivity and specificity of the CK20 RT-PCR [7]. CK20 is an epithelial cell intermediate filament protein confined to the gastrointestinal epithelium, cutaneous Merkel cells, and the urothelium as well as most of their neoplastic derivates. Considering that CK20 is absent in peripheral blood, bone marrow, and lymph nodes, it qualifies as a reliable marker for the detection of disseminated BCa cells [8].

The current study has analysed CK20 detection in bone marrow from a cohort of 51 BCa patients undergoing long-term observation for disease outcome following RC. This study presents the 5- and 7-yr survival rates of CK20-positive and CK20-negative BCa patients. In a multivariate analysis, the prognostic significance of CK20-positive disseminated bladder tumour cells was determined.

2. Patients and methods

2.1. Patient population

In a prospective study, we analysed bone marrow samples from 51 BCa patients (42 male, 9 female; 35–86 yr of age [median: 69]) with high-risk non–muscle-invasive- or muscle-invasive urothelial carcinoma (pT1/carcinoma in situ pN0 cM0 G3 to pT4a pN2 cM0 G3) undergoing RC between August 1998 and July 2002. Inclusion criteria were met by 51 patients, who provided written consent in accordance with the ethical guideline of the Christian-Albrechts University in Kiel, Germany. Standard RC included bilateral lymph node dissection with the templates of the external and common iliac lymph node group as well as the obturator region. BCa patients with histopathologically positive lymph node disease (pN1/pN2) were treated within 8 wk following RC by adjuvant chemotherapy. The schedule comprised three cycles of gemcitabine (1000 mg/m^2 on days 1, 8, and 15) and cisplatin (70 mg/m^2 on day 2) in a 4-wk setting. Patient characteristics and histopathology are summarised in Table 1.

2.2. Bone marrow sampling and qualitative reverse-transcriptase polymerase chain reaction

Preoperative bone marrow samples were collected from all 51 cystectomised patients in a standardised procedure. A Jamshidi needle (MD TECH, Gainesville, FL, USA) was passed through the incision into the bone marrow compartment from both anterior iliac crests, and bone marrow samples were aspirated into heparin tubes (10 ml) prior to RC [7]. Bone marrow samples were immediately processed by Ficoll-isopaque density gradient centrifugation to isolate mononuclear blood cells, allowing the separation of red blood cells and granulocytes from mononuclear blood

Table 1 – Patient demographics

Tumour stage				Total no. of patients (*n*)	No. of CK20-negative patients (%)	No. of CK20-positive patients (%)
All stages				51	35 (68.6)	16 (31.4)
pT1/pCIS	pN0	M0	G3	11	7 (63.6)	4 (36.4)
pT2	pN0	M0	G2/3	16	12 (75.0)	4 (25.0)
pT3	pN0	M0	G2/3	11	7 (63.6)	4 (36.4)
pT4a	pN0	M0	G2/3	3	2 (66.7)	1 (33.3)
pT2-T4a	pN1/N2	M0	G2/3	10	7 (70.0)	3 (30.0)
Age, yr						
Median				68.9	68.9	70.7
Range				34.8–86.0	54.8–79.9	34.8–86.0
Gender, no. (%)						
Male				42 (82.4)	28 (80.0)	14 (87.5)
Female				9 (17.6)	7 (20.0)	2 (12.5)

CK20 = cytokeratin-20; CIS = carcinoma in situ.

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cells [9]. Finally, mononuclear blood cells were lysed with RNAzol (WAK-Chemie Medical GmbH, Bad Homburg, Germany) [7,9]. RNA isolation and the CK20 RT-PCR protocol were performed for all patients as described previously [7]. Briefly, total RNA was isolated from mononuclear blood cells using the guanidinium thiocyanate-phenol-chloroform single-step isolation method.

The qualitative CK20 PCR was performed as a nested PCR, using the same primer pairs for both PCR reactions. Primers were synthesised by MWG-Biotech (Ebersberg, Germany). The primer combinations CK20-A sense, 5′-CGGCGGGGACCTGTTTGT-3 and CK20-B antisense, 5′-CAGTGTTGCCCAGATGCTTGTG-3 were used, resulting in a 485-bp product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control (GAPDH sense primer, 5′-CCAGCCGAGCCA-CATCGCTC-3 and GAPDH antisense primer, 5′-ATGAGCCCCAGCCTTCTC-CAT-3), resulting in a 359-bp product.

2.3. Follow-up of bladder cancer patients and clinical outcome

Follow-up of all patients were conducted postoperatively at 4-mo intervals for the first 2 yr, at 6-mo intervals the third and fourth year, and annually thereafter based on computed tomography or magnetic resonance tomography scans. Clinical tumour progression was defined by the presence of local tumour relapse and/or at least one measurable visceral or lymph node lesion as well as bone metastases by bone scan. The median follow-up for 27 of 51 patients who were still alive is 64 mo (range: 39–108). The median follow-up of all 51 patients was 50 mo (range: 1–108). Outcomes were measured by time to clinical progression as well as TSS and OS. Progression-free survival (PFS) was defined as the time from cystectomy to the first documented clinical progression or until last follow-up if the patient had not experienced a clinical recurrence. Patients who died before clinical progression were censored at the time of death. All deaths were discriminated between the tumour-specific event and death by other causes. Patients who are still alive were censored at the date of the last contact.

2.4. Statistical analysis

PFS, TSS, and OS from the time of surgery were defined as end points for this analysis. Distribution of event times was calculated with the univariate product-limit method by Kaplan and Meier [10]. The significance of prognostic variables was tested in a univariate manner with the log-rank test. Simultaneous effects of multiple factors were estimated by multiple regression analysis using the Cox proportional hazards model in a forward-selection strategy [11]. All reported p values were based on two-sided tests, and the threshold for significance was 0.05. Statistical analyses were performed with the SPSS v.14 (SPSS, Chicago, IL, USA).

3. Results

3.1. Histopathologic staging and analysis of clinical survival

Postoperative pathologic staging of 51 patients included 41 patients (80.4%) without evidence of lymph node involvement and 10 patients (19.6%) with lymph node–positive disease (Table 1). For all 51 patients, the 7-yr PFS, TSS, and OS were 56.3%, 62.5%, and 56.6%, respectively. Positive lymph node disease was associated with a significantly higher progression rate and worse TSS ($p < 0.001$). PFS and TSS for patients with lymph node–positive disease at 7 yr were both 20.0%. In contrast, BCa patients with a negative lymph node status displayed a 7-yr PFS of 65.6% as well as a 7-yr TSS of 73.9%. The hazard rate for disease progression in the lymph node–positive group was 3.76 (95% confidence interval [CI], 1.66–8.53) and for TSS 3.85 (95% CI, 1.67–8.83) compared to the lymph node–negative group.

3.2. Cytokeratin-20 detection in bladder cancer patients

In total, CK20-positive cells were detected in 16 of 51 (31.4%) preoperatively collected bone marrow samples from BCa patients of all stages. The frequency of CK20-positive bone marrow samples was not significantly associated with higher tumour stage as determined by a Kruskal-Wallis test. According to tumour classification, CK20 was positive in bone marrow from 4 of 11 patients (36.4%) with tumour stage pT1/pCispN0M0G3, 4 of 16 patients (25%) with pT2pN0M0G2/3, 5 of 14 patients (35.7%) with pT3/pT4apN0M0G2/G3, and 3 of 10 patients (30%) with lymph node metastases (pT2–pT4apN1/pN2M0G2/G3). Table 1 comprises the CK20 status in bone marrow according to tumour stage.

3.3. Cytokeratin-20 survival analysis

The PFS rate was significantly shorter ($p = 0.007$) in the CK20-positive group, with a 7-yr survival of 35.2% compared to CK20-negative patients with 64%. The median PFS time was 13.5 mo for CK20-positive BCa patients and >68.6 mo for the CK20-negative group (Fig. 1a). The 7-yr TSS rate for patients with CK20-negative bone marrow was 70.2%. In contrast, the CK20-positive group showed a 7-yr TSS rate of 46.9% ($p = 0.012$). The median TSS time was 19.4 mo for CK20-positive patients, whereas the CK20-negative group has not reached median survival after >97 mo (Fig. 1b). Furthermore, CK20-positive patients had a significantly shorter 7-yr OS rate of 37.5% than the CK20-negative group, which had an OS rate of 65.7% ($p = 0.006$). The median OS time was 18.4 mo for CK20-positive BCa patients and >97 mo for the CK20-negative group (Fig. 1c). The hazard rate for disease progression in the CK20-positive group versus the CK20-negative group was 3.02 (95% CI, 1.34–6.83), for TSS 2.89 (95% CI, 1.25–6.71), and for OS 2.91 (95% CI, 1.36–6.27). Table 2 summarises the survival analyses according to CK20 status in bone marrow. When assessing the possible independent effects of baseline factors such as gender, age at surgery, T classification, lymph node status, tumour differentiation, postoperative chemotherapy, and CK20 status on the risk for all three survival end points using multivariate Cox regression analysis, the only significant predictors were lymph node–positive disease and CK20 status in bone marrow (Table 3).

To further substantiate CK20 as an independent prognostic factor for bladder tumour patients without lymph node–positive disease (pN0), a subgroup analysis was performed for all 41 pN0 patients (Table 4). CK20-positive patients with pN0 status had a significantly shorter 7-yr PFS rate of 44.1% than the CK20-negative group, with 73.1% ($p = 0.009$; Fig. 2a). The TSS rate was significantly shorter in the CK20-positive group, with a 7-yr survival of 58.7%

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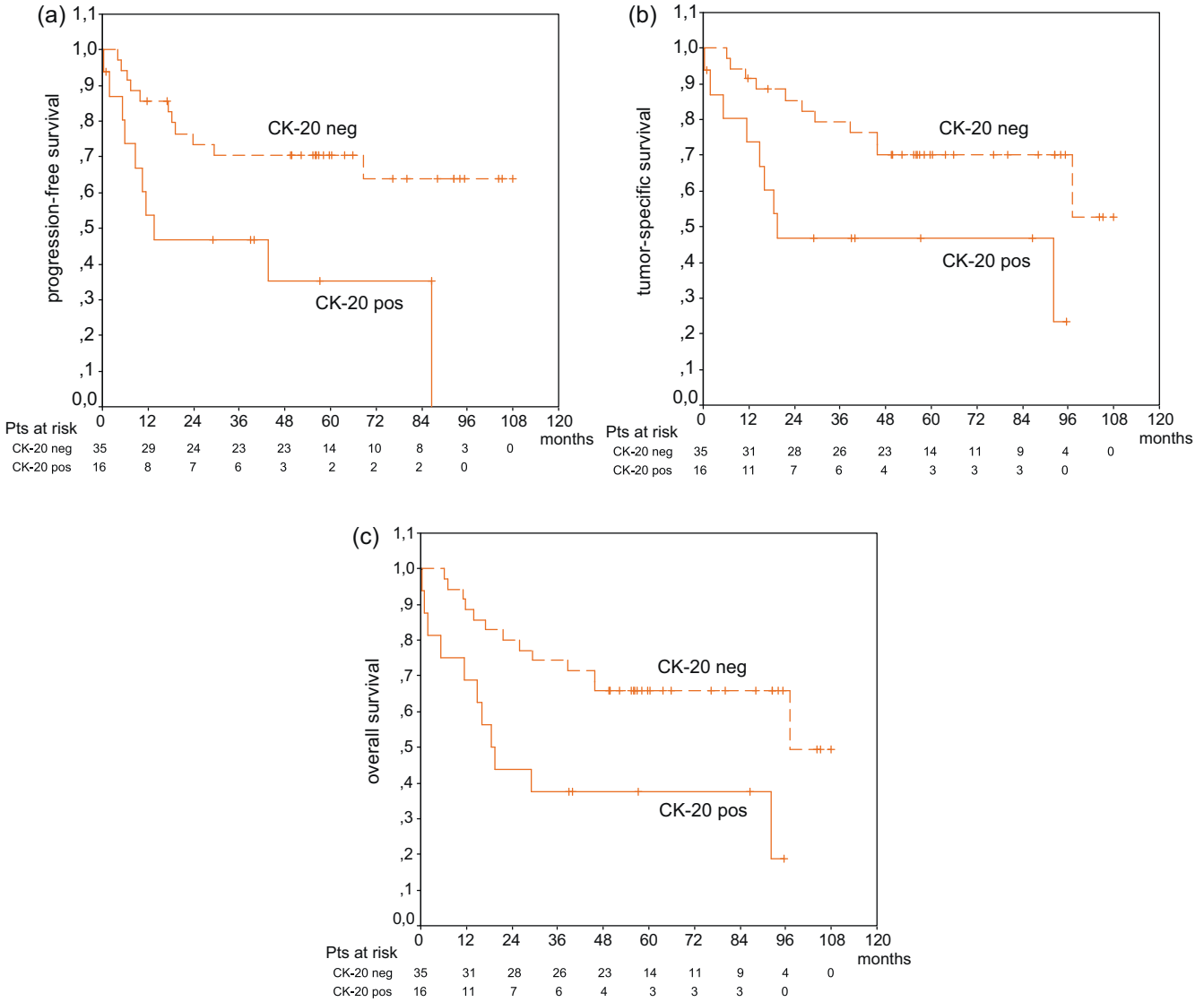

Fig. 1 – (a) Progression-free survival, (b) tumour-specific survival, and (c) overall survival depending on cytokeratin-20. PFS = progression-free survival; CK20 = cytokeratin-20; TSS = tumour-specific survival; OS = overall survival.

Table 2 – Survival analyses in all patients ($n = 51$)

	CK20 negative ($n = 35$)	CK20 positive ($n = 16$)
PFS		
Median time to progression, mo (95% CI)	>68.6	13.5 ± 17.0 (0.0–46.8)
HR (95% CI)	3.02 (1.34–6.83), $p = 0.007$	
Censored observations	68.6% ($n = 24$)	37.5% ($n = 6$)
	Estimated probability ± SE (%) of remaining event free	
5-yr PFS	70.41 ± 7.88	35.16 ± 14.02
7-yr PFS	64.01 ± 9.41	35.16 ± 14.02
TSS		
Median time to death by disease, mo (95% CI)*	>97.0	19.4 ± 26.7 (0.0–71.6)
HR (95% CI)	2.89 (1.25–6.71), $p = 0.012$	
Censored observations	68.6% ($n = 24$)	43.8% ($n = 7$)
	Estimated probability ± SE (%) of remaining event free	
5-yr TSS	70.17 ± 7.92	46.88 ± 12.89
7-yr TSS	70.17 ± 7.92	46.88 ± 12.89
OS		
Median time to death, mo (95% CI)*	>97.0	18.4 ± 3.4 (11.9–25.0)
HR (95% CI)	2.91 (1.36–6.27), $p = 0.006$	
Censored observations	62.9% ($n = 22$)	31.3% ($n = 5$)
	Estimated probability ± SE (%) of remaining event free	
5-yr OS	65.71 ± 8.02	37.50 ± 12.10
7-yr OS	65.71 ± 8.02	37.50 ± 12.10

PFS = progression-free survival; CK20 = cytokeratin-20; CI = confidence interval; HR = hazard ratio; SE = standard error; TSS = tumour-specific survival; OS = overall survival.

Table 3 – Multivariate Cox regression analysis (forward selection modus)*

Variable	Coefficient (ß)	p value	Exp(B) hazard rate	95% CI
PFS				
Positive lymph node	1.546	0.001	4.692	1.832–12.016
CK20 positive	1.199	0.010	3.317	1.330–8.271
TSS				
Positive lymph node	1.717	<0.001	5.568	2.068–14.997
CK20 positive	1.322	0.009	3.751	1.395–10.087
OS				
Positive lymph node	1.352	0.004	3.864	1.546–9.660
CK20 positive	1.266	0.005	3.548	1.472–8.548

PFS = progression-free survival; CK20 = cytokeratin-20; TSS = tumour-specific survival; OS = overall survival.
* Parameters entered in the analysis: age at surgery, gender, lymph node status, tumour differentiation, T classification, CK20 status, and postoperative chemotherapy.

compared to CK20-negative patients, with 81.3% ($p = 0.025$; Fig. 2b). The 7-yr OS rate for patients with CK20-positive bone marrow was 46.2% versus 75.0% in the CK20-negative group ($p = 0.014$; Fig. 2c).

In summary, a stratification of 41 lymph node–negative patients by CK20 expression status in bone marrow revealed significant differences with respect to all three survival end points. CK20 provides independent prognostic information, particularly in pN0 patients. Further comparison of pN+CK20-negative versus pN+CK20-positive patients revealed a significant difference for TSS and OS ($p = 0.047$) but not for PFS ($p = 0.262$). Analyses of pN+CK20-negative versus pN0 CK20-positive patients showed no significant differences in PFS ($p = 0.744$) as well as TSS ($p = 0.627$) or OS ($p = 0.999$).

4. Discussion

The aim of this study was to assess the prognostic value of detecting DTCs in bone marrow from BCa patients following RC. In the past decade, PCR-based assays became the most widely used alternative to immunocytochemical assays [12]. The main advantage of RT-PCR for the detection of DTCs at the single-cell level is the availability of various primers for almost every gene of interest, the high sensitivity, the observer-independent result, and the combination of a multiple molecular marker system [12]. However, the clinical relevance of PCR-based techniques for DTC detection in bone marrow is still questionable without long-term follow-up analysis for BCa patients.

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Table 4 – Survival analyses in pN0 patients ($n = 41$)

	CK20 negative ($n = 28$)	CK20 positive ($n = 13$)
PFS		
Median time to progression, mo (95%CI)	>68.6	43.7 ± 34.1 (0.0–110.6)
HR (95% CI)	3.83 (1.39–10.55), $p = 0.009$	
Censored observations	78.6% ($n = 22$)	46.2% ($n = 6$)
	Estimated probability ± SE (%) of remaining event free	
5-yr PFS	81.25 ± 7.58	44.06 ± 16.58
7-yr PFS	73.12 ± 10.29	44.06 ± 16.58
TSS		
Median time to death by disease, mo (95% CI)	>97.0	92.2 ± 54.4 (0.0–198.9)
HR (95% CI)	3.32 (1.14–9.65), $p = 0.025$	
Censored observations	78.6% ($n = 22$)	53.9% ($n = 7$)
	Estimated probability ± SE (%) of remaining event free	
5-yr TSS	81.25 ± 7.58	58.74 ± 14.19
7-yr TSS	81.25 ± 7.58	58.74 ± 14.19
OS		
Median time to death, mo (95% CI)	>97.0	28.9 ± 26.5 (0.0–80.9)
HR (95% CI)	3.15 (1.24–7.96), $p = 0.014$	
Censored observations	71.4% ($n = 20$)	38.5% ($n = 5$)
	Estimated probability ± SE (%) of remaining event free	
5-yr OS	75.00 ± 8.18	46.15 ± 13.83
7-yr OS	75.00 ± 8.18	46.15 ± 13.83

CK20 = cytokeratin-20; PFS = progression-free survival; CI = confidence interval; HR = hazard ratio; TSS = tumour-specific survival; OS = overall survival.

In the present study, we show for the first time long-term results, analysing 7-yr survival rates of BCa patients in correlation with their CK20 status in bone marrow. We demonstrate that BCa patients with CK20-negative status displayed a significantly longer PFS, TSS, and OS rate in contrast to the CK20-positive group. In addition, multivariate analysis revealed that CK20 expression and lymph node status provided independent prognostic information with respect to all three survival end points. Moreover, stratifying bladder tumour patients by lymph node disease, CK20 provides independent prognostic information in pN0 patients as well as in the lymph node–positive group. Survival plots of pN+CK20-negative versus pN0 CK20-positive patients show no significant statistical survival differences ($p > 0.05$). Our data strongly support the use of PCR-based detection of DTCs in bone marrow to identify a high-risk subgroup in BCa patients with pN0 status.

In our study, the detection rate of CK20-positive BCa cells in bone marrow was 31.4%. Notably, the CK20 status by qualitative RT-PCR in bone marrow isolates was independent of tumour stage. These data are in accordance with the recent report from Ribal and colleagues [13]. Although BCa patients with early tumour stage displayed CK20-positive bone marrow status, a subgroup of patients with histopathologically proven, overt lymph node metastasis was missing CK20-positive cells. This contradiction could be explained by the parallel progression model, in which the metastatic capacity is gained early during primary tumour development. The parallel progression model is characterised by moving the starting point of metastatic dissemination to the clinically undetectable phase of cancer growth years before diagnosis of a primary tumour [14,15].

In contrast, a considerable percentage of negative findings of DTCs in bone marrow from bladder tumour patients with overt metastasis were observed. This phenomenon could be expounded by changes in cancer cell phenotype between epithelial to mesenchymal states, defined as epithelial–mesenchymal transition (EMT), which plays a key role in tumourigenesis. Tumour cells in the EMT state employ early developmental processes accompanied by changes in the expression of their molecular repertoire [16]. Loss of CK20 expression in BCa cells of individual tumours could be one EMT transdifferentiation program. The development of a tumour marker set by codetection of CK20 and other molecular markers might improve this observed lack of sensitivity.

Although PCR-based detection of DTCs in bone marrow is the most commonly used technique within the past two decades, controversies addressed are mainly attributed to a lack of methodologic uniformity comprising standardised sample collection, preparation procedure, and reaction conditions for RT-PCR [17,18]. Our previous study, which was initiated in 1998, has focused on these technical pitfalls and proposed new approaches in the standardisation of the CK20 RT-PCR detection assay [7]. From today's view, qualitative PCR has the disadvantage that the calculation of cut-off level regarding transcript level and clinical follow-up is not possible. An enhanced technical improvement would be the use of a quantitative PCR that enables us to quantify the absolute number of copies of a specific CK20 sequence and would provide a more detailed analysis and stratification of the amount of tumour cells in the bone marrow of BCa patients.

Critical comments have been raised that bone marrow aspiration is not considered a convenient and practical

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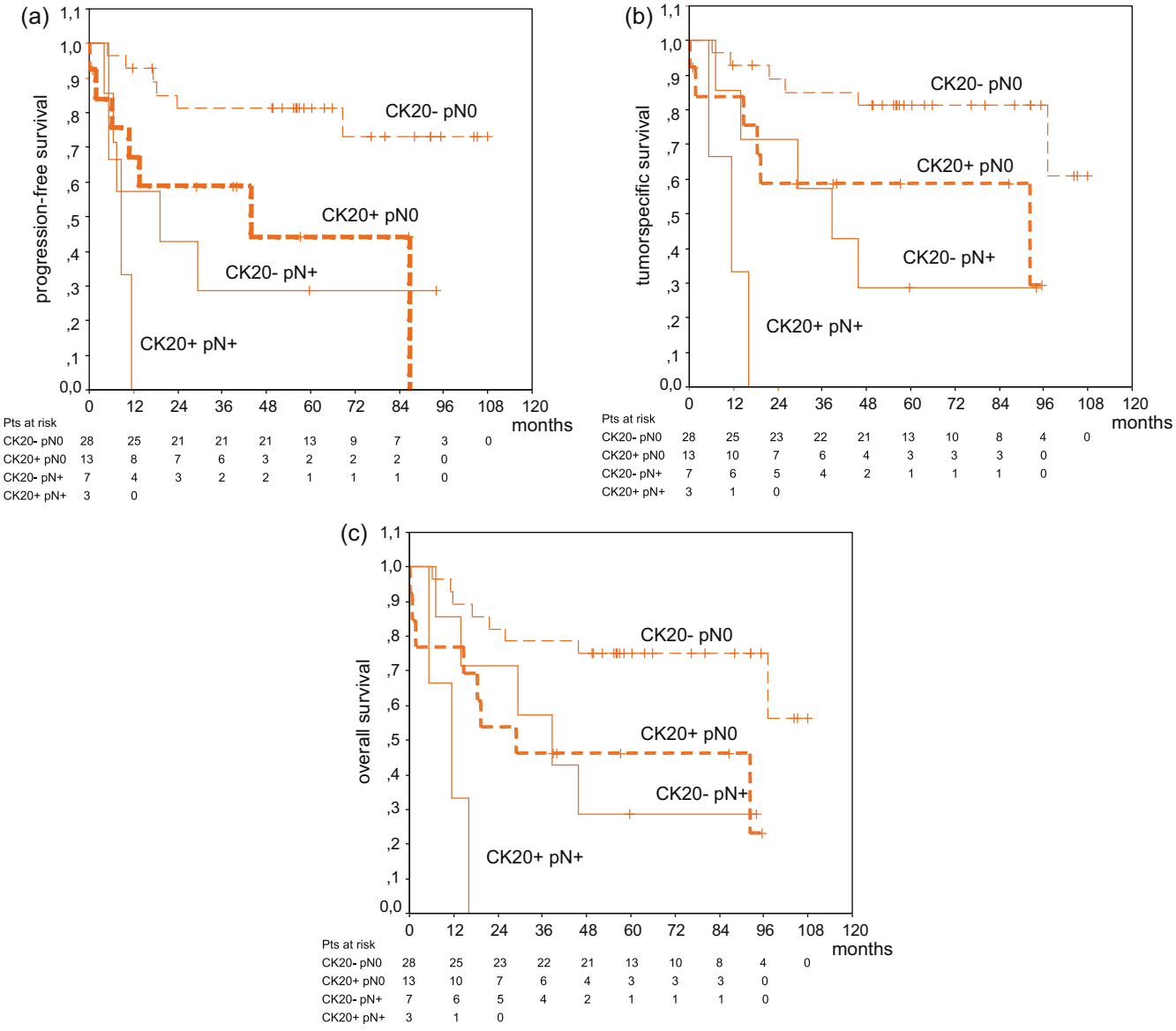

Fig. 2 – (a) Progression-free survival, tumour-specific survival, and overall survival stratified by cytokeratin-20 status in bone marrow samples and lymph node involvement.
PFS = progression-free survival; CK20 = cytokeratin-20; TSS = tumour-specific survival; OS = overall survival.

procedure for patients. An alternative detection system for disseminating tumour cells might be displayed by detecting circulating tumour cells (CTC) in peripheral blood (eg, with the CellSearch system [Veridex, Raritan, NJ, USA]) [19]. Although the detection of CTCs in the peripheral blood of breast cancer patients with metastases was highly predictive for PFS and OS, the prognostic relevance of CTCs in the peripheral blood of patients with early-stage cancer without overt metastases has only been proven by very few studies [20,21]. A study from Guzzo and co-workers investigated the prognostic significance of CTCs in peripheral blood from BCa patients with organ-confined disease prior to RC. They concluded that the CTC status is not likely to be a clinically useful parameter for directing therapeutic decisions in patients with muscle-invasive BCa [22].

Although our long-term analysis by RT-PCR provides a promising approach to identify a high-risk subgroup of BCa patients, a consensus concept for standardised detection and enrichment of DTCs on the basis of a multicentric clinical trial is warranted. In collaboration with the research network German Bladder Cancer Association, which was founded in May 2008 by urologists, scientists, and pathologists, we have recently developed a multicentre study with a novel quantitative, real-time multiplex PCR containing the marker CK20, mucin 7, mucin 5AB, and uroplakin-II to enhance the sensitivity and specificity of PCR-based detection of disseminated bladder tumour cells in bone marrow [23]. To determine the prognostic value of DTCs, a larger cohort of 400 BCa patients is planned in this multicentre

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study; at the time of writing, 120 patients had entered this trial for further molecular analysis.

5. Conclusions

BCa patients with disseminated CK20-positive cells in bone marrow represent a high-risk subgroup reflected by an unfavourable outcome in the long-term analysis. A multicentre clinical trial is warranted to confirm CK20 status in bone marrow as an independent prognostic factor that might provide additional information for clinical management of individual patients with BCa.

Author contributions: M. Retz had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Retz, Lehmann.
Acquisition of data: Rotering.
Analysis and interpretation of data: Retz, Lehmann, Nawroth.
Drafting of the manuscript: Retz, Buchner, Nawroth.
Critical revision of the manuscript for important intellectual content: Stöckle, Buchner, Gschwend.
Statistical analysis: Lehman.
Obtaining funding: Stöckle, Gschwend.
Administrative, technical, or material support: Rotering, Lehmann.
Supervision: Retz.
Other (specify): None.

Financial disclosures: I certify that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

Funding/Support and role of the sponsor: The Hensel Foundation of Kiel, Germany, and the Gruber Foundation of Munich, Germany sponsored the design and conduct of this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.eururo.2010.12.014.

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Please cite this article in press as: Retz M, et al. Long-term Follow-up of Bladder Cancer Patients with Disseminated Tumour Cells in Bone Marrow. Eur Urol (2010), doi:10.1016/j.eururo.2010.12.014