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Dorothea Weckermann · Friedhelm Wawroschek
Gunnar Krawczak · Karl-Heinz Haude · Rolf Harzmann

Does the immunocytochemical detection of epithelial cells in bone marrow (micrometastasis) influence the time to biochemical relapse after radical prostatectomy?

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Abstract The detection of cytokeratin-positive bone marrow cells has been considered a prognostic factor in numerous malignant tumors. We investigated whether this was also valid for localized prostate cancer. Bone marrow aspirates were taken prior to radical prostatectomy from 169 consecutive patients with pT1/2 pN0 G1–3 adenocarcinoma of the prostate. The immunocytochemical detection of cytokeratin no. 18 (CK 18)-positive cells using monoclonal antibody CK 2 was interpreted as micrometastasis. Repeat marrow aspirations were performed at 6 months postoperatively and once a year thereafter. The patients were re-examined over a period of at least 10 and a maximum of 72 months (median 32 months). An increase in prostate specific antigen ≥0.5 ng/ml was considered a biochemical "relapse". One hundred and fifty-four patients had evaluable bone marrow aspirates, of which 74.7% were CK 18-negative and 25.3% positive. The latency period for biochemical relapse was 1481 days (median) in the CK 18-negative group and 1106 days (median) in the CK 18-positive group. This difference was not statistically significant. The CK 18-positive aspirates ($n = 39$) showed one positive cell in 20 cases, two positive cells in 8 and three or more positive cells in 11 cases. The preoperative number of cells had no statistically significant effect upon the onset of biochemical relapse. Only patients with three or more CK 18-positive cells tended to have a poorer prognosis. One hundred and thirteen patients had evaluable bone marrow aspirates pre- and postoperatively. Postoperative persistence or occurrence of CK 18-positive cells did not affect the outcome of the disease. The detection of CK 18-positive cells in bone marrow does not influence the prognosis of patients with localized prostate cancer within a period of 32 months (median). Solely a subgroup of patients showing a large preoperative number of CK 18-positive cells seems to tend to an unfavorable course of the disease. Thus, further studies are necessary aiming at a more detailed characterization of these cells.

Key words Micrometastasis · Organ-confined prostate cancer · Immunocytochemistry · Cytokeratin · Monoclonal antibody · Bone marrow examination

D. Weckermann (✉) · F. Wawroschek
G. Krawczak · R. Harzmann
Urologische Klinik, Zentralklinikum,
Stenglinstrasse 2, D-86156 Augsburg, Germany

K.-H. Haude
Klinik für Anästhesiologie und operative Intensivmedizin,
Zentralklinikum, Augsburg, Germany

Introduction

Disseminated micrometastases at the time of surgery of the primary tumor are considered the major problem in terms of a curative therapy. Especially in patients with colorectal tumors and breast cancer an early dissemination of tumor cells often determines the patients' destiny. Since novel radiological techniques fail to provide sufficient information in the early stages of tumor dissemination, immunocytochemical and immunohistochemical methods have recently been applied.

Due to the fact that cells containing cytokeratin no. 18 do not normally occur in bone marrow, their presence in patients with clinically locoregional carcinoma is regarded as micrometastatic spread. Since, in 1980, Sloane et al. [21] succeeded in proving the existence of disseminated tumor cells in the marrow of breast cancer patients, numerous studies followed which correlated the proof of epithelial cells in bone marrow with conventional risk factors and/or follow-up data, thereby arriving at the statement that these cells are of prognostic relevance for different types of tumors [4]. We investigated whether this was also valid for localized prostate cancer.

Patients and methods

After informed consent had been obtained, bone marrow aspirates from both sides of the anterior iliac crest were taken immediately

prior to radical prostatectomy from 169 patients with pT1/2 pN0 G1–3 prostate cancer between October 1992 and December 1997. The median age of the patients was 66 years (range 51–78 years). The presence of cytokeratin no: 18 (CK 18)-positive cells was interpreted as micrometastasis.

The patients were followed over a period of at least 10 and a maximum of 72 months (median 32 months), including prostate specific antigen (PSA) levels, digital rectal examination (DRE) and transrectal ultrasound (TRUS). Depending on the PSA levels, CT or MRI scans were performed on a facultative basis, whereas total-body bone scanning was obligatory. An increase in PSA ≥0.5 ng/ml was considered a biochemical "relapse". Patients with PSA persistence were also considered. Repeated bone marrow aspirations were performed 6 months postoperatively and once a year thereafter.

The aim of this study was to find out whether men with preoperatively positive CK 18 findings developed a biochemical "relapse" more often than those with negative findings. In this context we investigated furthermore whether the quantity of CK 18-positive cells was clinically relevant. By means of repeated bone marrow aspirations we were able to monitor the individual course of micrometastasis and to analyze the significance of postoperative persistence, disappearance or occurrence of CK 18-positive cells.

Bone marrow aspiration and immunocytochemistry

Bone marrow aspirates of 6–8 mL were taken from both sides of the iliac crest in syringes containing 1000 U heparin/ml marrow. Marrow fat was separated by centrifugation and the red blood cells still contained in suspension were lysed by addition of a buffer solution. Mononuclear cells were collected from the interphase after density centrifugation through Ficoll-Paque (Seromed, Berlin, Germany). The total number of marrow cells was then determined using a Neubauer counting chamber. The non-vital cells were marked by means of trypan blue staining. An average number of 1×10^6 bone marrow cells was centrifuged on glass slides in a cytocentrifuge. After overnight air-drying the slides were either immediately fixed and stained or stored at a minimum temperature of −80°C. The staining took place under humid room conditions at room temperature, using PBS (Phosphate buffered saline, pH 7.6; own production) as a medium for incubation. Non-specific bonding sites were blocked by addition of a 10% antibody serum (AB-Serum, Biotest, Dreieich, Germany).

For immunostaining the monoclonal antibody against CK 18 was used at a concentration of 0.05 μg/ml (Klon CK 2, Boehringer Mannheim, Mannheim, Germany). Subsequently, the antibody reaction was developed with alkaline phosphatase with a polyvalent rabbit anti-mouse Ig antiserum (rabbit anti-mouse IgG, Dako, Hamburg, Germany) and preformed complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase antibodies (APAAP-Komplexe, Dako, Hamburg, Germany), according to the technique described by Cordell et al. [2]. Cells containing CK 18 were stained red with neofuchsin and naphthol-AS-biophosphate after inhibition of endogenous phosphatase by preincubation with levamisole. The cells were washed three times between each of the steps.

Negative control of the staining One isotype control per side of the iliac crest was carried out with each staining in order to exclude nonspecific reactions. This was achieved by an inhibition of the CK 18-bonding locations with mouse IgG1 (MOPC 21).

Positive control of the staining A cell line of colorectal adenocarcinoma, the cells of which express CK 18, served as a positive control.

Negative control of the method The negative control was carried out in 10 patients without any underlying malignant disease. Nine patients suffered from urolithiasis and one had impaired micturition. Four of these patients were female and six were male. The age range of the patients was 27–46 years (median 34 years).

Positive control of the method The positive control was performed in patients suffering from prostate cancer with osseous metastasis ($n = 43$).

Statistical analysis

The frequency of biochemical "relapse" in CK 18-positive and CK 18-negative patients was evaluated by means of life table analysis and a Gehan test (generalized Wilcoxon test). The defined level of significance was 0.05. In order to detect the clinical effects of the quantity of CK 18-positive cells prior to radical prostatectomy, we applied life table analysis with a Gehan test and also – due to the small number of patients – Kaplan-Meier analysis with log-rank, Breslow and Tarone-Ware tests.

By means of these tests we also registered the latency period of biochemical "relapse" in men with pre- and postoperatively negative cytological findings, in patients with preoperatively negative and postoperatively positive findings, in those with preoperatively positive and postoperatively negative bone marrow results as well as in the group with CK 18-positive cells pre- and postoperatively. Here, the defined level of significance was also 0.05.

Results

Of the overall group with organ-confined prostate cancer ($n = 169$), 15 puncture specimens were technically not evaluable. Of the remaining 154 specimens 115 (74.7%) were CK 18-negative and 39 (25.3%) were positive. The median latency period of biochemical "relapse" (PSA ≥0.5 ng/ml) was 1481 days for the CK 18-negative group and 1106 days for the positive group. This difference was not statistically significant (Fig. 1).

Patients with positive bone marrow findings ($n = 39$) showed one CK 18-positive cell in 20 cases, two in 8 and three or more in 11 cases, each per 1 to 2×10^6 mononuclear cells (Table 1). According to the life table analysis, the median latency period of biochemical "relapse" was 1145 days regarding the patient group with one CK 18-positive cell, 975 days in the group with two positive cells and 908 days in those patients having three or more CK 18-positive cells (Fig. 2). Table 2 presents the results of the Kaplan-Meier analysis. Probably due to the small number of patients, the differences between these groups are not statistically significant.

The bone marrow aspirations were done again at defined intervals in order to follow the individual course of micrometastasis and to determine its influence on the rate of biochemical "relapse". Of the 169 patients with organ-confined prostate cancer, 11 preoperative bone marrow aspirates were not evaluable, as were 41 postoperative aspirates, and in four cases neither pre- nor postoperative aspirates were evaluable. Consequently, only 113 patients were eligible for this study.

Fifty-nine of these 113 patients had negative pre- and postoperative findings, 23 had negative preoperative and positive postoperative results, 18 showed positive pre-

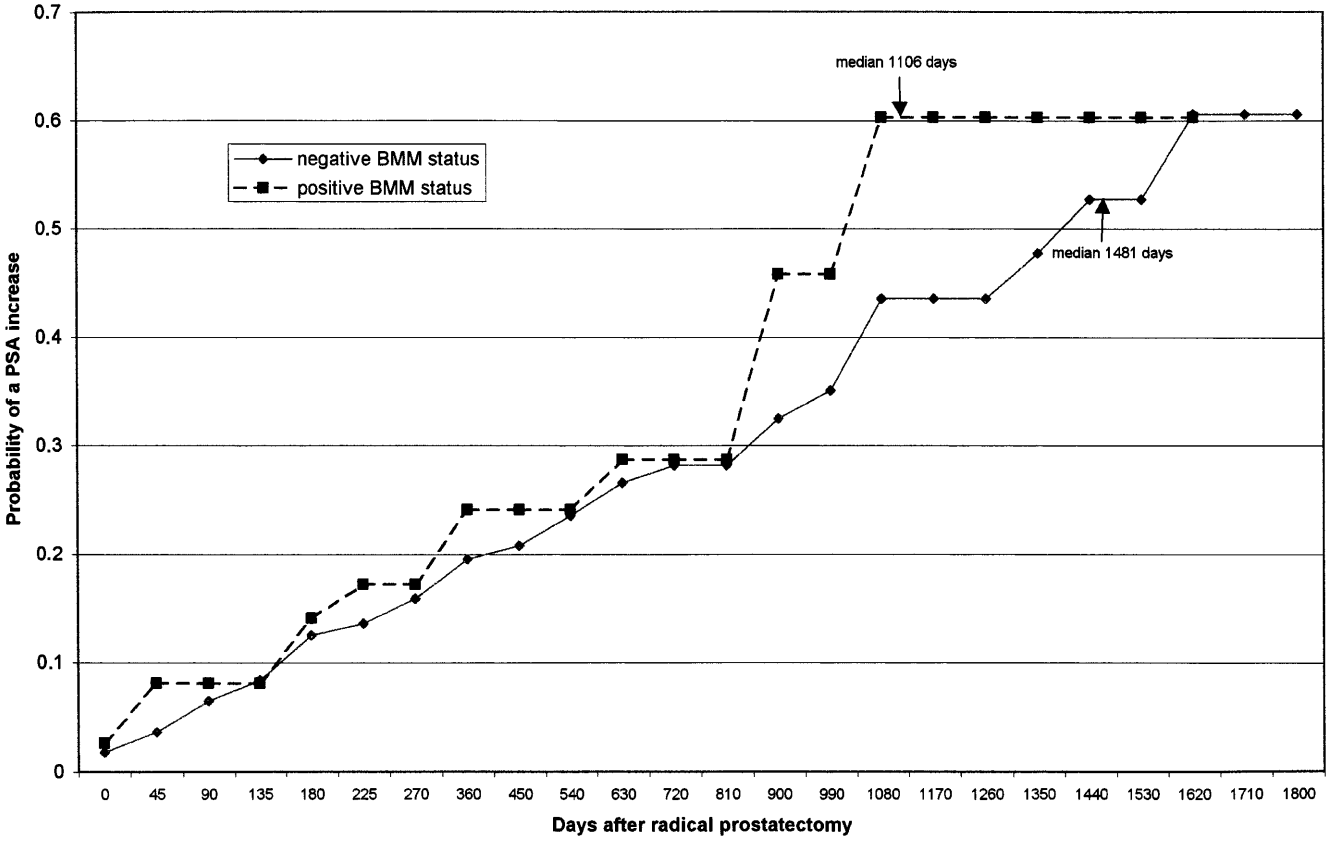

Fig. 1 Probability of biochemical "relapse" after radical prostatectomy dependent on the preoperative bone marrow micrometastases (BMM) status (life table analysis)

Table 1 Number of CK 18-positive cells in organ-confined prostate cancer (pT1/2 pN0, $n = 169$) prior to radical prostatectomy

No. of patients	No. of CK-positive bone marrow cells
115	0
20	1
8	2
2	3
2	4
1	5
2	6
2	8
1	21
1	51
15	Not evaluable

operative and negative postoperative bone marrow findings and in 13 patients positive pre- and postoperative bone marrow findings were recorded. The results of the life table analysis are depicted in Fig. 3. The median latency period of PSA increase (life table and Kaplan-Meier analysis) is shown in Tables 3 and 4. According to the life table and Kaplan-Meier analysis, the differences among the four patient groups were not statistically significant. The group with positive preoperative and negative postoperative results ($n = 18$) had a PSA increase in only one case.

One aspirate was not evaluable in the control group without underlying malignant disease ($n = 10$), eight specimens were CK 18-negative and one was CK 18-positive (one cell/1 $\times$ 10^6 mononuclear cells). Patients with prostate cancer metastasizing to the skeleton showed positive findings in 22 of 40 evaluable aspirates (55%), some expressing large numbers of cells (1 to 1000 cells/1 to 2 $\times$ 10^6 mononuclear cells) or cell clusters.

Discussion

Numerous studies have described the immunocytochemical detection of disseminated bone marrow cells containing cytokeratin in women and men with different epithelial tumors [6, 11, 18, 20]. Most of these refer to breast cancer, which has a median prevalence of bone marrow micrometastasis of 35% [1, 3, 4, 8]. Other publications concentrate on colorectal tumors, carcinoma of the lung, head, neck, stomach, pancreas and esophagus. The median prevalence of bone marrow micrometastasis in these tumors reaches 38.5% [4]. For localized prostate cancer only data pertinent to relatively small series are available. In these patients, a prevalence of bone marrow micrometastasis of between 13% and 54.5% is reported [5, 7, 9, 17]. Our own data on 287 patients with localized and lymphatically spread

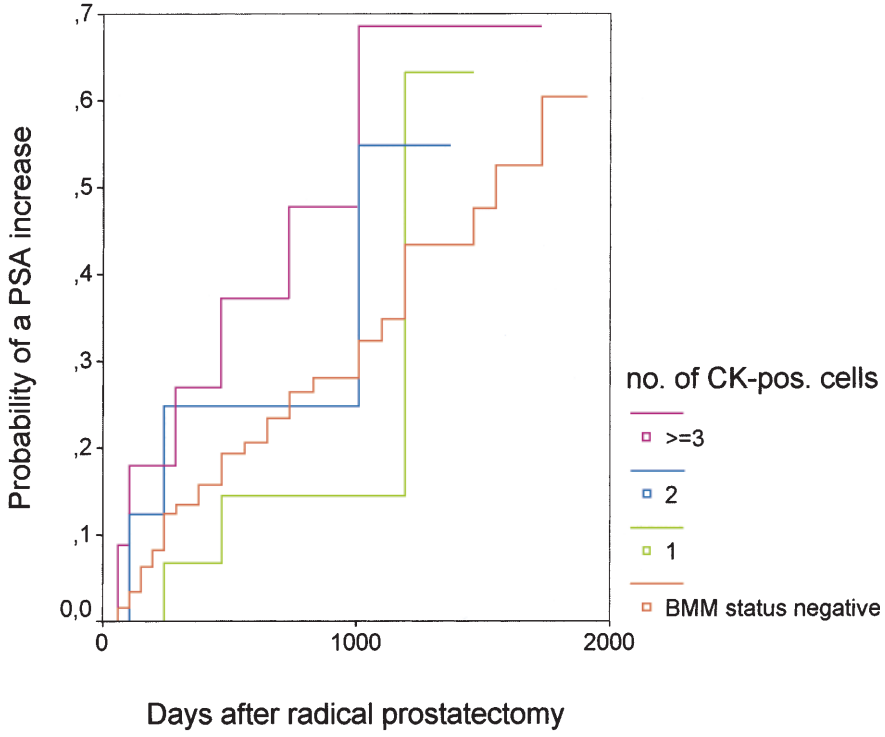

Fig. 2 Probability of biochemical "relapse" dependent on the preoperative quantity of cytokeratin 18 (CK 18)-positive cells (life table analysis) (*BMM* bone marrow micrometastases)

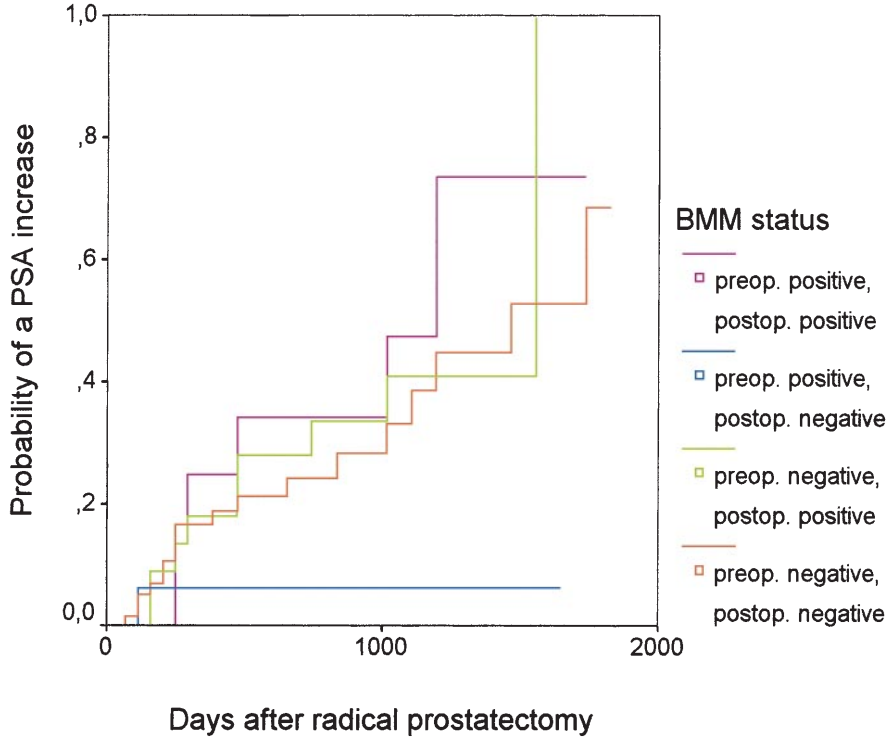

Fig. 3 Life table analysis regarding the prostate specific antigen (PSA) increase after radical prostatectomy dependent on the pre- and postoperative bone marrow micrometastases (BMM) status

Table 2 Median time to prostate specific antigen (PSA) increase dependent on the number of CK-positive cells in bone marrow prior to radical prostatectomy according to Kaplan-Meier analysis (*BMM* bone marrow micrometastases)

Negative BMM status	(n = 115)	1499 ± 218 days	(1072; 1926)
One CK-positive cell	(n = 20)	1158 ± 52 days	(1055; 1261)
Two CK-positive cells	(n = 8)	958 ± 553 days	(0; 2041)
Three or more CK-positive cells	(n = 11)	917 ± 357 days	(217; 1617)

Table 3 Median time to PSA increase dependent on pre-and postoperative bone marrow micrometastases status according to life table analysis

All patients (n = 113)	1407 days (≈3.8 years)
Pre- and postoperatively negative (n = 59)	1408 days (≈3.8 years)
Preoperatively negative/postoperatively positive (n = 23)	1454 days (≈4.0 years)
Preoperatively positive/postoperatively negative (n = 18)	1530 days (≈4.2 years)
Pre- and postoperatively positive (n = 13)	1089 days (≈3.0 years)

Table 4 Median time to PSA increase dependent on pre- and postoperative bone marrow micrometastases status according to Kaplan-Meier analysis

Pre- and postoperatively negative (n = 59)	1412 ± 182 days (1055; 1769)
Preoperatively negative/postoperatively positive (n = 23)	1499 ± (*) days ((*); (*))
Preoperatively positive/postoperatively negative (n = 18)	Only one PSA increase
Pre- and postoperatively positive (n = 13)	1090 ± 418 days (272; 1908)

(*) Median = last PSA increase

prostate cancer revealed positive findings in 23.7% (7.3% of these aspirates were not evaluable) (unpublished data).

Many studies have reported a correlation between the presence of CK-positive cells and established risk factors in malignant tumors of the genitourinary tract [5, 10]. However, our data from a large series did not show any correlation between the established risk factors (grading, T-stage, infiltration of the seminal vesicles, apex or perineurium, ploidy, preoperative PSA level, lymphangiosis carcinomatosa and N-stage) and positive preoperative bone marrow findings. Neither were we able to detect any interdependency between the quantity of CK 18-positive cells and the established risk factors (unpublished data).

Many epithelial tumors (breast cancer, carcinoma of the stomach and the esophagus, colorectal carcinoma, non-small-cell lung cancer and tumors of the head and neck) have been proven to have a significantly poorer prognosis in cases with positive preoperative bone marrow findings in contrast to those with negative preoperative findings [4, 6, 14, 20]. We wanted to determine whether this was the case for organ-confined prostate cancer. Bone marrow aspirates were taken from 169 patients immediately prior to and periodically after radical prostatectomy. Our data revealed that patients with positive preoperative findings did not have to expect a significantly poorer prognosis, i.e. a higher biochemical ''relapse'' rate, compared with those with negative preoperative marrow findings. Neither did either the postoperative persistence or the de novo detection of CK-positive cells have any prognostic value. The only remarkable fact was a worse prognosis in those patients with a large quantity of CK 18-positive cells prior to radical prostatectomy compared with those showing smaller quantities or negative findings. Because of the relatively small group this difference was not statistically significant.

The immunocytochemical detection of cells containing cytokeratin is explicitly defined by the evidence of epithelial cells in nonepithelial tissue such as bone marrow, not allowing any further statement as regards the origin, vitality and oncogenic potential of these cells. Thus, the clinical relevance of these findings is indeed doubtful. The specificity of the immunocytochemical detection of CK-positive cells is focused on, among others, by a study dealing with a large series of patients. In this study only six of 215 patients (2.8%) without a detectable malignant tumor had one CK 18-positive cell [18].

In our own control group eight bone marrow aspirates were negative and one was not evaluable. One patient suffering from urolithiasis had one CK 18-positive cell per 1×10^6 mononuclear cells. On the other hand we were able to detect positive bone marrow aspirates containing very large quantities of CK 18-positive cells (1 to 1000 cells per 1 to 2×10^6 mononuclear cells) in 22 of 40 patients suffering from prostate cancer metastasizing to the skeleton.

The immunocytological detection of epithelial cells in bone marrow suggests a rather low sensitivity in the case of small quantities of cells, since it ought to be difficult to identify one single CK-positive cell in a pool of 1 to 2 million cells. The down-regulation of CK 18 in bone marrow micrometastasis is another reason for false-negative results [16].

Published data regarding a small series of patients provide further information on the origin of the CK 18-positive cells in prostate cancer patients, since in 5 of 13 patients coexpression of CK 18 and PSA was present [19]. The oncogenic potential of CK-positive cells was confirmed by double staining [14, 15, 20]. Furthermore, chromosome aberrations and a loss of HLA antigens in micrometastases could be proven [12, 13].

Surprisingly, own data do not, at present, confirm the perception of other authors in terms of an effect of CK-positive cells before and after radical prostatectomy on the development of a biochemical ''relapse''. We were unable to confirm the role of disseminated CK-positive bone marrow cells as an (independent) prognostic factor. The number of CK 18-positive cells merely suggests a poorer outcome of the disease: of the 11 patients who had three or more CK 18-positive cells prior to radical prostatectomy, one developed a skeletal metastasis 1 year after surgery and six showed biochemical relapse. Four of these patients remained free of relapse.

The results must be extrapolated with caution, as the group with organ-confined prostate cancer is relatively small (n = 169) and the median postoperative follow-up period of 32 months might not be sufficient. Furthermore, the rate of biochemical ''relapse'' is relatively

high, which could result from the former use of rather restrictive pelvic lymphadenectomy, solely removing the lymph nodes of the obturator fossa and the area of the external iliac vessels. In addition, the removed lymph nodes were not examined immunohistochemically (understaging).

The available data regarding the immunocytochemical detection of bone marrow cells containing cytokeratin in prostate cancer are controversial and not comparable, since they are derived from studies with heterogeneous patient groups and different follow-up periods. Apart from that, the results of bone marrow examinations are influenced by the monoclonal antibody, the number and volume of bone marrow aspirates, and the number of evaluated bone marrow cells per aspirate. We can, at present, state that CK 18-positive bone marrow cells are detectable in 24.2% (own data) to 54.5% of patients with localized prostate cancer, that these findings are reproducible, and that the cells containing cytokeratin originate at least partly from the prostate or correlate with findings in the primary tumor. In organ-confined prostate cancer the detection of CK 18-positive cells does not alter the prognosis of the disease, over a median follow-up period of 32 months, though those patients with large preoperative quantities of cells obviously have a poorer prognosis.

We therefore conclude that further studies should aim at proving the statement that CK-positive cells are vital cells originating from the prostate which may lead to clinically relevant metastasis.

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